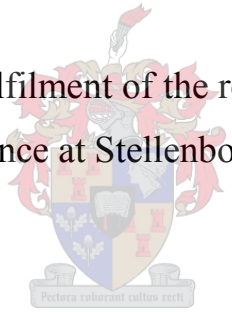
Molecular aspects of exploited sharks in South Africa: multiple paternity and identification of novel molecular markers

by

Charné Rossouw



Thesis presented in partial fulfilment of the requirements for the degree of Master of Science at Stellenbosch University

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DECLARATION

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December 2015

SUMMARY

Sharks have existed for the past 400 million years and play an important role in the oceanic ecosystem as they occupy the upper categories of the food web. Since the 1920s they have been fished on a commercial scale as the demand for shark related products increased. This placed considerable pressure on shark populations, resulting in a global decline of many populations and an increased risk of population extinction. As the genetic diversity of a population determines it's resilience to changing environmental factors, including such information has become paramount for short and long-term management and conservation of individual species. This study therefore aimed to add to the growing body of biological and genetic data by investigating mating strategy through assessing the presence of multiple paternity (MP) in three commercially important shark species: the common smoothhound *Mustelus mustelus*, dusky shark *Carcharhinus obscurus* and the scalloped hammerhead shark *Sphyrna lewini*; and by identifying potential microsatellite and single nucleotide polymorphism (SNP) markers in two of the species, *M. mustelus* and *C. obscurus*, through the use of Next-Generation Sequencing (NGS) platforms.

The first aim of this study was achieved by cross-amplifying microsatellite markers developed in closely related species to the study species. A total of 22 microsatellite markers were initially tested on four litters of *M. mustelus* and *C. obscurus* in order to determine the most informative markers for parentage analysis. Reduced marker panels of five to six microsatellites were selected and parentage analysis in GERUD and COLONY revealed the presence of MP in all three species. *Mustelus mustelus* had the highest frequency of MP (67%), followed by *S. lewini* (46%) and *C. obscurus* (35%).

The second aim of this study entailed reduced genome sequencing of one *M. mustelus* and one *C. obscurus* individual using the HiSeq Illumina and Ion Proton platforms, respectively.

For *M. mustelus*, 51,5 million reads with an average read length of 250bp were obtained, whereas *C. obscurus* yielded 27,6 million reads with an average read length of 213bp. Contigs were constructed for both species in order to search for perfect repeat motifs. In total, 2 700 and 1 255 microsatellite-containing regions were identified for *M. mustelus* and *C. obscurus* respectively. In order to search for SNP-containing regions, both sample species were aligned to previously assembled scaffolds of the ghost shark *Callorhinchus milii*, which served as a reference genome. After quality filtering, only 767 SNP-containing regions were identified for *M. mustelus*, whereas the identification of potential SNPs for *C. obscurus* was not successful.

The insights gained into the mating strategies of *M. mustelus*, *C. obscurus* and *S. lewini* as well as the identification of potential species-specific molecular markers add to the growing body of information and genetic resources available for exploited species. In future, this information could be used for further molecular assessment of shark populations and a more intergrated approach to conservation and management of these already vulnerable sharks.

OPSOMMING

Haaie bestaan reeds vir die afgelope 400 miljoen jaar en speel 'n belangrike rol in die oseane se ekosisteme omdat hulle die boonste kategorie van die voedselweb in beset neem. Sedert die 1920's word haaie op 'n kommersiele vlak gevis soos die aanvraag vir haai verwante produkte toeneem. Dit het aansienlike druk op haai populasies geplaas wat 'n globale afname van verskeie populasies se getalle tot gevolg gehad het en hul kans op uitwissing vergroot het. Omdat die genetiese diversiteit van 'n populasie die weerstandigheid van 'n populasie teen veranderinge in die omgewing bepaal, het dit toenemend belangrik geword om sogenaamde inligting in te sluit vir kort en langtermyn bestuur en bewaring van die spesie. Hierdie studie het daarom gepoog om tot die groeiende liggaam van biologiese sowel as genetiese data by te dra deur die voortplantingsstrategieë te ondersoek in terme van die teenwoordigheid van veelvoudige vaderskap (VV) in drie kommersiele belangrike haai spesies: die algemene hondhaai *Mustelus mustelus*, donkerhaai *Carcharhinus obscurus* en die skulprand-hammerkophaai *Sphyrna lewini* te asseseer; en deur moontlike mikrosatelliet en enkel-nukleotied-polimorfiese merkers te identifiseer in *M. mustelus* en *C. obscurus* deur die gebruik van volgende-generasie volgorde bepalings platforms (NGS).

Die eertse doelwit van die studie is bereik deur die kruisamplifisering van mikrosatelliet merkers wat ontwikkel is in nabyverwante spesies tot die studiespesies. 'n Totaal van 22 mikrosatelliet merkers is op vier werpsels *M. mustelus* en *C. obscurus* getoets om die mees informatiewe mikrosatelliete vas te stel vir ouerskaptoetse. Verkleinde merkerpanele van vyf tot ses mikrosatelliete is geselekteer en ouerskap analises in GERUD en COLONY het die teenwoordigheid van VV in al drie spesies vasgestel. *Mustelus mustelus* het die hoogste frekwensie van VV gehad (67%), gevolg deur *S. lewini* (46%) en *C. obscurus* (35%).

Die tweede doelwit van die studie het die verkleinde genoom volgorde bepaling van een *M. mustelus* en een *C. obscurus* individu behels deur gebruik te maak van beide 'n HiSeq Illumina en Ion Proton platvorm. Vir *M. mustelus* is 51,5 miljoen lesings met 'n gemiddelde lengte van 250bp en 27,6 miljoen lesings met 'n gemiddelde lengte van 213bp vir *C. obscurus* verkry. *Contigs* is vir beide spesies gebou om vir perfekte mikrosatellietstreke te soek. In totaal is 2 700 en 1 255 mikrosatellietstreke is vir *M. mustelus* en *C. obscurus* geidentifiseer. Beide spesies is tot die voorafontwikkelde *Callorhinchus milii* stelasies belyn (wat as 'n verwysingsvolgorde gedien het) om vir SNP-streke te soek. Na kwaliteitsfiltering is slegs 767 SNP-streke vir *M. muselus* geidentifiseer terwyl SNP-streek identifikasie vir *C. obscurus* nie suksesvol was.

Die insig wat oor die voortplantingsstrategieë vir *M. mustelus*, *C. obscurus* en *S. lewini* verkry is asook die identifisering van moontlike spesie-spesifieke molekulêre merkers sal bydra tot die groeiende liggaam van data en genetiese bronne wat beskikbaar is vir hierdie uitgebuite haai spesies. In die toekoms kan hierdie inligting vir die verdere molekulêre assessering van haai populasies gebruik word om sodoende ‘n meer geïtegreerde benadering tot die bewaring en bestuur van hierdie reeds kwesbare haaie te weeg te bring.

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Assessing multiple paternity in three exploited shark species occurring in southern Africa: *Mustelus mustelus*, *Carcharhinus obscurus* **and** *Sphyrna lewini*

Bioinformatic analyses

I hereby declare that all bioinformatics analyses were preformed by me and not by an external bioinformatician

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LIST OF ABBREVIATIONS

%	Percentage
>	Greater than
®	Registered trademark
°C	Degrees Celsius
µg	Microgram
µL	Microliter
µM	Micromole
3’	Three prime
5’	Five prime
$	Dollar
A	Adenine
ARC	Agricultural Research Council
BLAST	Basic Local Alignment Search Tool
bp	Base Pair
C	Cytosine
CAF	Central Analytical Facility
CITES	International Trade in Endangered Species of Wild Fauna and Flora
CTAB	Cetyl trimethylammonium bromide
DAFF	Department of Agriculture, Forestry and Fisheries
DNA	Deoxyribonucleic Acid
dNTP	Deoxyribonucleotide Triphosphate
EDTA	Ethylenediaminetetraacetic acid ($C_{10}H_{16}N_2O_8$)
EST	Expressed Sequence Tags
EEZ	Economic Zone

F	Forward Primer
FAO	United Nations Food and Agricultural Organization
G	Guanine
GB	Gigabyte
HPC	High-Performance Computing
ID	Identifier
IPOA	International Plan of Action
IUCN	International Union for Conservation of Nature
KZN	KwaZulu-Natal
Mb	Megabyte
$MgCl_2$	Magnesium Chloride
mM	Millimole
MP	Multiple Paternity
MPA	Marine Protected Area
mtDNA	Mitochondrial DNA
N	Unidentified Nucleotide
NCBI	National Center for Biotechnology Information
ng	Nanogram
ng/µL	Nanogram per Microlitre
NGS	Next-Generation Sequencing
NH_4OAc	Ammonium acetate
NPOA	National Plan of Action for the Conservation and Management
PCR	Polymerase Chain Reaction
PIC	Polymerase Chain Reaction
QTL	Quantitative Trait Locus
R	Reverse Primer
RAD	Restriction site Associated DNA sequencing

SNP	Single Nucleotide Polymorphism
SSG	Shark Specialist Group
SSR	Simple Sequence Repeats
T	Thymine
TAC	Total allowable catch
Taq	*Thermus aquaticus* DNA Polymerase
TWG	Technical Working Group
VNTR	Variable Number Tandem Repeat

CHAPTER 1

Literature review

1.1 Chondrichthyans and global concern

Chondricthyan fishes, who have existed for the past 400 million years in diverse marine ecosystems, are a group of a 1 000 species consisting of sharks, skates and chimeras (Musick *et al*. 2000; Molina *et al*. 2012; Worm *et al*. 2013). Since their arrival, sharks have occupied the upper categories of the food web, with most sharks playing an important role in coastal as well as oceanic ecosystems' top-down control (Carrier *et al*. 2010; Biery & Pauly 2012; Dulvy *et al*. 2013). By influencing behaviour of meso-consumers and mortality rates, sharks help structure marine communities (Molina *et al*. 2012). If sharks were to be removed from the ecosystem it could lead to a knock-on effect resulting in a change of prey community composition (Stevens *et al*. 2000; Carrier *et al*. 2010). Removal of larger apex predator sharks could lead to disruptions of both non-shark fisheries and ecosystems (Worm *et al*. 2013). Even the discard of remains after shark finning has an effect on the amount of available food for scavenging species (Stevens *et al*. 2000). The removal of sharks can also have a socio-economical influence as many communities in both the developed and developing countries rely on sharks as a source of protein and various other products. If these resources were to become scarcer, fisheries would have to look to already limited fish resources to fill the niche (Simpfendorfer *et al*. 2011).

Even though sharks enjoy a high trophic level status, they face many threats such as habitat destruction, overexploitation due to fisheries, climate change and pollution (Simpfendorfer *et al*. 2011; Dulvy *et al*. 2013). Fisheries can affect the size structure and composition of populations through targeted fishing, non-target species being caught as by-catch as well as habitat alteration (which could lead to a change in overall biomass). Life-history traits, physical changes in the environment as well as trophic interactions among species determine a species response to fishing pressures (Bianchi *et al*. 2000). Sharks follow a K selected life history strategy which means that in comparison to teleost fishes, sharks and rays produce fewer eggs, are less fertile, have relatively slow growth rates and reach sexual maturity at a much later age (Musick *et al*. 2000; Stevens *et al*. 2000). Life history traits are manifested by the available energy within the environment as well as the interactions within the

environment that affect the energy allocation. This energy is assigned to various biological processes within the organism. The available energy is dependent on the ecosystem's primary productiveness (which is influenced by habitat) whereas the biological interaction strength is dependent on characteristics within the ecosystem such as population diversity and structure (Garcia *et al*. 2008). This K selected life history strategy in turn makes sharks vulnerable to overfishing due to low resilience to fishing mortality and slow intrinsic increase rates (Musick *et al*. 2000; Stevens *et al*. 2000). Low productivity such as late sexual maturity, small litters, long inter-birth intervals and slow growth rates affects how well a species is able to adapt to increased mortality rates and this in turn makes sharks extremely vulnerable to extinction.

Due to their K selected life history strategies and an increase in demand for shark related products, shark populations are declining rapidly on a global and regional scale. Sharks can only withstand a modest level of fishing before population decline sets in (Musick *et al*. 2000; Dulvy *et al*. 2013; Worm *et al*. 2013). Not only do sharks mature much later than teleost fishes, they have some of the highest levels of maternal investment, longest gestation periods and very few offspring when compared to other marine taxa which results in populations growing at a much slower rate (Simpfendorfer *et al*. 2011; Dulvy *et al*. 2013). The mode of reproduction can also be linked to vulnerability to extinction. Studies have shown vulnerability is smallest in oviparous species, heightened in lecitho-trophic viviparous species and most severe in adelphophagic, histotrophic, oophagic and placental viviparous species. Females which have a matrotrophic mode of reproduction tend to reproduce less often and give birth to smaller litter sizes as the cost of energy to reproduce is high (Garcia *et al*. 2008). How a species responds to fishing pressures is mediated by its density-dependent response, productivity and vulnerability (Musick *et al*. 2000).

To date, a third of all threatened shark species are still being subjected to targeted fishing. Figure 1.1 depicts the severity of endangerment for the 1 041 species of shark being monitored by the IUCN. Although no shark species has been driven to extinction, several populations of angel sharks, sawfish and skates have gone extinct locally or regionally (Dulvy *et al*. 2013). It is troublesome that nearly 50% of fished species are listed as data deficient. Due to sharks not being viewed as economically important until recently, limited baseline data has been collected on landings or species identification. This is due to research priorities being linked to a particular species' economic value (Stevens *et al*. 2000).

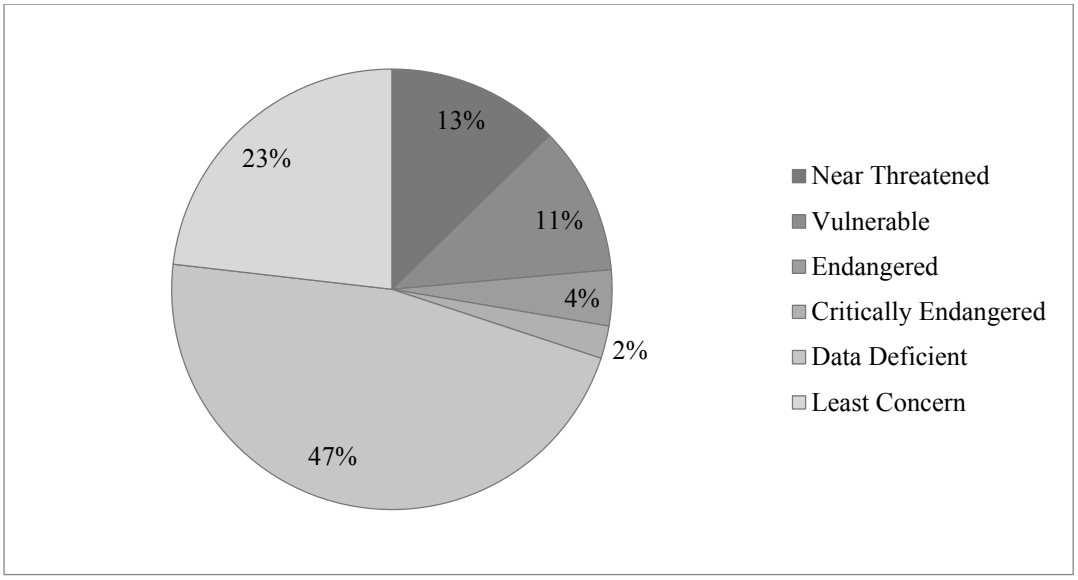

Figure 1.1: Level of endangerment of the 1 041 species monitored by IUCN taken from Dulvy *et al.* (2013).

1.2 Chondrichthyan fisheries

On a global scale, chondrichthyan fisheries have been increasing steadily since the 1980s with catches totalling 760 000 tons in the year 2000 (Stevens *et al*. 2000). This increase in chondricthyan fisheries was brought on by the shift in demand for shark related products from liver oil, meat, leather, jaws, teeth and fins (Biery & Pauly 2012). The demand for shark fin was driven largely by the shark fin markets in Asia, which used fins as a delicacy in their soups in the more expensive Asian restaurants (Musick *et al*. 2000; Dulvy *et al*. 2013). Some of the most valuable fins are harvested from guitarfish, sawfish and wedgefish (Dulvy *et al*. 2013). Shark fins can reach a price of up to $30 a pound, which has led to a worldwide increase in the practice of finning (Musick *et al*. 2000). Finning is the act of processing sharks at sea where heads, fins and intestines are removed to reduce the amount of storage space needed in order to maximize profit (Stevens *et al*. 2000; Pinhal *et al*. 2008; Biery & Pauly 2012). This can be considered as a wasteful practice as the sharks are not utilized to their maximum potential (Biery & Pauly 2012). In 2010, approximately 9 500 metric tons of shark fins were exported to Hong Kong with the fin trade remaining unregulated in more than 80 countries (Dulvy *et al*. 2013).

In 1999, shark conservation took another step forward with the founding of the International Plan of Action for Sharks (IPOA-Sharks) which focused not only on management and

conservation, but also on enhancing monitoring and data collection. This was followed by authorities such as: Australia, Canada, Europe and the United States placing a ban on finning in 2000. Regardless of these countries introducing legislations against finning, it still continues in many other unlegislated areas of the oceans (Worm *et al*. 2013). Since the implementation of fishing quotas and management action plans, to date only 10 species of sharks and rays enjoy protection by CITES, with an additional seven species being added to the list to be considered (Dulvy *et al*. 2013). Some of these species that are already under protection are the basking shark *Cetorhinus maximus*, the great white shark *Carcharodon carcharias* and whale sharks *Rhincodon typus* (Worm *et al*. 2013). By the early 1990s the Shark Specialist Group (SSG) was formed by the Species Survival Commission of the IUCN which focussed on the conservation and management of sharks on a global scale. A review on the status and trade of sharks by the International Trade in Endangered Species of Wild Fauna and Flora (CITES) was mandated in 1994. This was also followed by the formation of the Technical Working Group (TWG) on sharks by the United Nations Food and Agricultural Organization (FAO) (Stevens *et al*. 2000).

Although legislations had been put in place to conserve Chondricthyan species, fishery landings increased from 120 000 tons in the 1950s to 400 000 tons in 1997. Following a similar trajectory to global fishing, this number decreased to 380 000 tons in 2010. From the 1990s to the present day, Chondricthyan landings have remained stable in America, Asia, Europe and Oceania but have increased in Africa. Figure 1.2 shows the trend of reported landings of Chondricthyan fishes over the last 60 years (Worm *et al*. 2013). In several cases Chondricthyans fisheries have increased to compensate for scarcer teleost fishes as well as increased restrictions on TACs of commercial species (Dulvy *et al*. 2013).

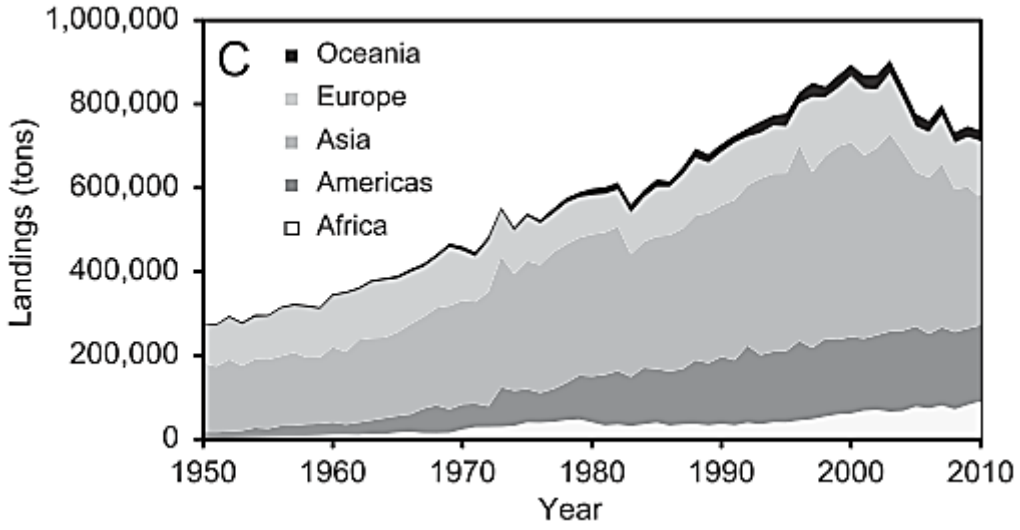

Figure 1.2: Trend of reported Chondricthyan landings from 1950 to the present (Worm *et al.* 2013).

Although landings have been kept stable since the 1990s, there has been a sharp increase in the trade of shark fins (Dulvy *et al*. 2013). The United Nations Food and Agriculture Organization (FAO) is responsible for receiving reports on all landed shark catches. Many catches (approximately 50%) are however not reported and instead discarded at sea after removing the fins (Stevens *et al*. 2000; Worm *et al*. 2013). This is due to sharks being seen as by-catch when fisheries target teleost species or due to countries not keeping adequate fishing records (Stevens *et al*. 2000). By-catch can be defined as the accidental capture of non-desirable species when using non-selective fishing gear (Molina *et al*. 2012). Most of the by-catch in fisheries occur when using long-lines, demersal trawlers or gillnets. Furthermore many shark species exhibit highly migratory natures, which place them outside the jurisdiction of a single country (Stevens *et al*. 2000).

Reported catches could represent only a portion of the total number of sharks caught and killed. It is estimated that roughly 61 million sharks are caught annually but the total amount of catches are more likely to be closer to 100 million individuals if unreported landed catches are factored into the equation. Furthermore, it is estimated that 227 000 tons of shark are being released alive after finning with 15% (34 000 tons) of these sharks suffering post-release mortality (Worm *et al*. 2013). This presents a problem as most fisheries aim to balance production and mortality. By not reporting by-catches or lumping by-catches into vague categories such as skates, rays and hammerheads, total mortality of these Chondricthyan species may be well above the rate of replacement (Stevens *et al*. 2000;

Maduna *et al*. 2014). It is estimated that only a third of reported catches specifies the species caught. This poor regulation and recordings of catches lead to a general lack of Chondrichthyan species-specific biological data. (Dulvy *et al*. 2013).

An estimated 66.9% of IUCN threatened species are in decline due to fisheries by-catch (Molina *et al*. 2012). Despite the regulations on finning practices in some countries, the problem remains and is far from solved. Instead fisheries have simply moved to unregulated areas in order to meet the demand for shark fins (Worm *et al*. 2013). Furthermore, finning complicates catch monitoring as shark bodies are discarded before they can be counted or weighed. Using only fins to calculate the number of landings may not be as accurate as being able to count and weigh the carcasses which could lead to inaccurate catch data. This in turn poses a threat to management as catch data is usually underestimated and fishing pressures are still poorly understood (Biery & Pauly 2012).

Another problem arises with fraudulent substitution since most shark products are already processed when purchased (von der Heyden *et al*. 2014). Less valuable shark species are being sold to consumers under false generic names. *Mustelus mustelus* common smoothhound is ranked as one of the top five commercial species in southern African waters (Serena *et al*. 2009). Investigations showed that in some cases products being sold as fresh smoothhound were in fact *Squalus* spurdogs, *Cirrhigaleus* dogfishes and *Prionace glauca* blue sharks, whereas frozen products were substituted with *Isurus oxyrinchus* mako or *Lamna nasus* porbeagle sharks (Renon *et al*. 2001; Barbuto *et al*. 2010). The mislabeling of shark products could compromise accurate data collection and in turn effective management efforts.

As sharks are both ecologically and socio-economically important it is essential to develop effective conservation and management programs to address the drastic decline in numbers (Simpfendorfer *et al*. 2011). To date, there is still no global action plan to ensure proper implementation of management and conservation mechanisms after 20 years of creating awareness of Chondricthyan population declines (Dulvy *et al*. 2013). Given that almost half of declining species are data deficient, assessment cannot be done to ensure proper integration with existing conservation and management of these species (Simpfendorfer *et al*. 2011). More research needs to be conducted in order to understand these species' vulnerability to being overfished as well as their role in the ecosystem in order to tailor management programs for these species. Developing genetic markers for these shark species will not only provide simple and flexible tests for species identification but will also

contribute to expanding our genetic resources on these shark species. These resources can be further used to better understand species' biology, ecology and spatial structure (DeSalle & Amato 2004), which in turn are essential in the development of improved conservation strategies for these species.

1.3 Regional chondrichthyan biodiversity & fisheries

South Africa has a coastline of roughly 3700km and 200 nautical miles of Exclusive Economic Zone (EEZ) (Nielsen & Martin 1996; Griffiths *et al*. 2010). Of South Africa's EEZ, only 1% falls under marine protection areas (MPAs). Globally, it has the third most biologically diverse marine life (approximately 12 900 species of marine biota) with coastal and offshore zones being grouped into nine marine bioregions (Griffiths *et al*. 2010). South Africa has an incredibly diverse shark, ray and chimaera biodiversity that includes representatives from all 10 orders of cartilaginous fishes, 44 of the 60 families (73%) and 100 out of 189 genera (53%). An estimated 15% of the world's shark species can be found around the southern African coastline with 27.1% of the Chondrichthyan species being endemic to this region (DAFF 2012). To date, approximately 204 species of chondrichthyans have been recorded in southern African waters, of which 117 are shark species, 69 skate and ray species and 8 chimaera species (Ebert & van Hees 2015). South Africa is a centre of endemism for a variety of chondrichthyans including catsharks (family Scyliorhinidae), finback catsharks (Proscylliidae), dogfish (Squaliformes), houndsharks (Triakidae), sawfishes (Pristiophoridae), skates (Rajoidei) and chimaeras (Chimaeriformes) (Compagno 1999). Despite this high level of shark biodiversity and endemism, our knowledge of South African sharks is relatively scarce (Bester-van der Merwe & Gledhill 2015). What is however known from historical and current catch data is that stock sizes are relatively small. A diverse range of habitats over a small spatial scale restricts distribution ranges within many species resulting in low abundance and pronounced vulnerability to over-fishing (Compagno 2002; DAFF 2012). There is evidence of declines from commercial catch data and observations from shark longline fishermen for many commercially important species in South Africa (da Silva & Bürgener 2007).

Fishing industries within South Africa took off in the 1950s with extensive developments in the pelagic fisheries, yielding 360 000 tons on average per annum (Geromont *et al*. 1999). In 2010, total catches within the South African commercial fishing sector amounted to roughly

600 000 tons with a fiscal value of approximately R6 billion with R3,2 million being made from exports (DAFF 2014). Figure 1.3 depicts the breakdown of the South African fishing sector as reported by Nielsen & Martin (1996) and DAFF (2012).

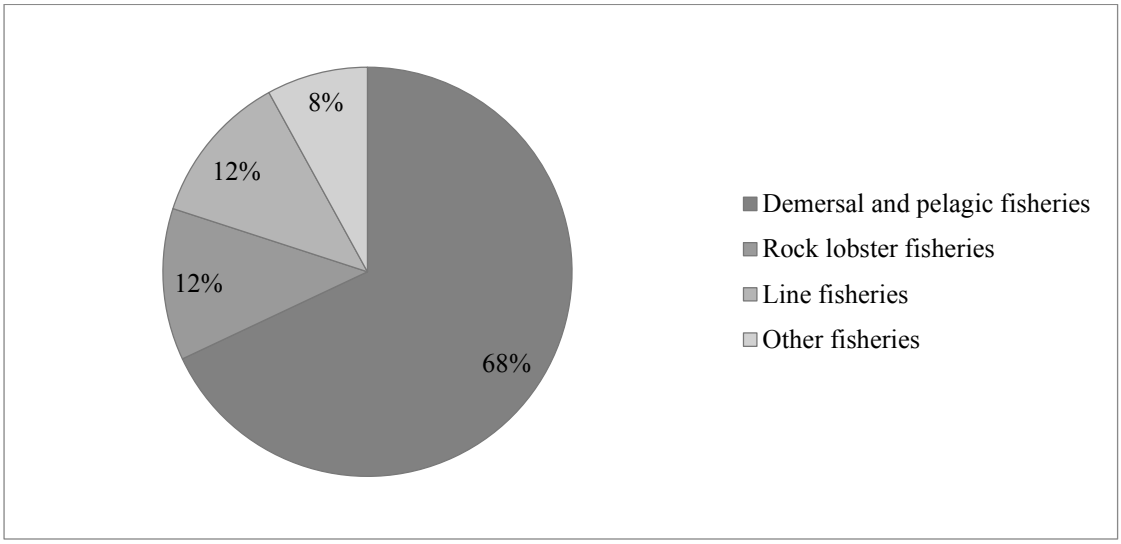

Figure 1.3: Breakdown of South African fishing sector constructed from Nielsen & Martin (1996) and Department of Agriculture, Forestry and Fisheries (2012).

The South African fishing industry supplies employment to primarily semi-skilled and unskilled workers, with the Western Cape at the heart of the fishing industry. While the East coast of South Africa has densely populated communities, it has fewer smaller commercial fisheries which lead to overfishing of the inshore resources (Griffiths *et al*. 2010; DAFF 2014). The industry employs approximately 27 000 labourers of which 16 000 are in the primary sector (utilizing 1 800 vessels) and the remainder in the secondary and tertiary sectors. A further 900 000 inhabitants are also involved in deep-sea, surf and shore angling (DAFF 2014).

It was not until 1988 that South Africa introduced the Sea Fisheries Act, which allowed the Environmental Affairs and Tourism Minister to control policies regarding the utilisation and conservation of South African marine resources (Nielsen & Martin 1996). This was followed by the first induction of Management procedures (MPs) in the early 1990s, which regulated the demersal and pelagic fisheries of hake, anchovy and sardines through regulations such as total allowable catches (TACs) (Geromont *et al*. 1999). To date, only 9% of coastal MPAs receive full protection with an uneven spread of MPAs across the nine marine bioregions. Less than 0, 2% of the offshore regions hold full conservation status (Griffiths *et al*. 2010).

A large number of chondrichthyans are caught in South Africa via directed and by-catch fisheries with approximately 49% of local species affected by nine different fisheries in South Africa (da Silva *et al*. 2015). The main species affected by the shark trade in South Africa include smoothhound sharks (e.g. *Mustelus mustelus, M. palumbes*), tope sharks *Galeorhinus galeus*, bronze whaler sharks *Carcharhinus brachyurus*, dusky sharks *Carcharhinus obscurus*, hammerhead sharks (e.g. *Sphyrna zygaena and S. lewini*), spotted gully sharks *Triakis megalopterus* and blacktip sharks *Carcharhinus limbatus*. Several other catsharks, skates and rays are also primarily caught as by-catch in South African waters. Combined with the life-history characteristics of these species (long generation time, late age at maturity, first age of reproduction), this makes these chondrichthyans highly vulnerable to over-exploitation (Bester-van der Merwe & Gledhill 2015).

1.4 Study species

In this study, the focus was primarily on species affected by fisheries in South Africa for which very little molecular resources are available such as the common smoothhound *Mustelus mustelus,* dusky shark *Carcharhinus obscurus* and scalloped hammerhead *Sphyrna lewini*. Of these study species, no species-specific molecular markers have been developed for either *Mustelus mustelus* or *Carcharhinus obscurus*. Fifteen microsatellite markers have previously been developed for *Sphyrna lewini* (Nance *et al*. 2009). No SNP markers have been developed for any of the study species. *Mustelus mustelus* (Figure 1.4) is a small coastal shark which can be found in the Mediterranean Sea as well as the eastern Atlantic Ocean from the British Isles to South Africa (Saïdi *et al*. 2009). It aggregates in large social groups often within enclosed bays and does not migrate across large distances (Smale & Compagno 1997). This in itself poses a problem as large amounts of sharks can be fished from a single population due to their aggregating behaviour. These sharks produce between 4-15 pups and are important in both commercial and artisanal fisheries. This is due to the ease with which this species is caught (they do not damage fishing gear and can be caught off small fishing boats or from the shore) (Smale & Compagno 1997). *Mustelus mustelus* is caught by shore based anglers, commercial trawlers as well as line-fish boats (Compagno 1984; Goosen & Smale 1997; Compagno *et al*. 2005). These sharks were generally not used for food consumption but have been increasingly fished since the 1980s for exportation, especially in the Western Cape in the absence of prime teleost fishes. This shark species is intensely fished

in St Helena Bay, Struis Bay as well as Saldanha Bay (Smale & Compagno 1997). *Mustelus mustelus* is listed as vulnerable by the IUCN with a decreasing population trend (Serena *et al*. 2009).

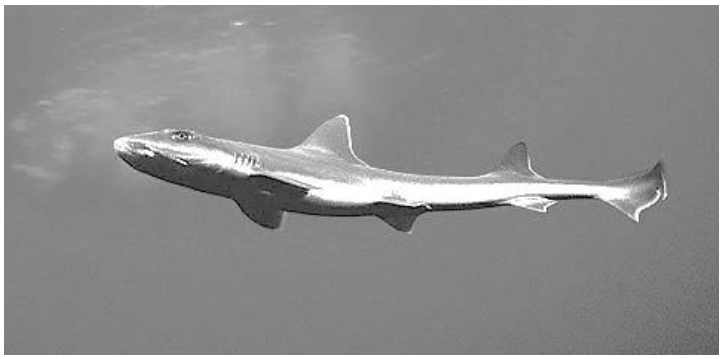

Figure 1.4: *Mustelus mustelus* shark.

The dusky shark *Carcharhinus obscurus* (Figure 1.5) is a large pelagic shark found globally in warm temperate and tropical waters from the Western Indian Ocean to South Africa. In South Africa, adults are common in water 200-400 meters deep with females showing site fidelity to nursery grounds in KwaZulu-Natal (KZN) (Dudley *et al*. 2005). *Carcharhinus obscurus* has been described as having a very slow growth rate and late sexual maturity (approximately 20 years), as well as a long gestation period (estimated 24 months) and very few pups (3-16) which make them especially vulnerable to overfishing (Hussey *et al*. 2009). Due to an increased demand for *C. obscurus* fillets since the year 2000, young *C. obscurus* have been targeted by recreational and commercial fisheries in KZN (Dudley & Simpfendorfer 2006). *Carcharhinus obscurus* has been listed as vulnerable by the IUCN with a deceasing population trend (Musick *et al*. 2009).

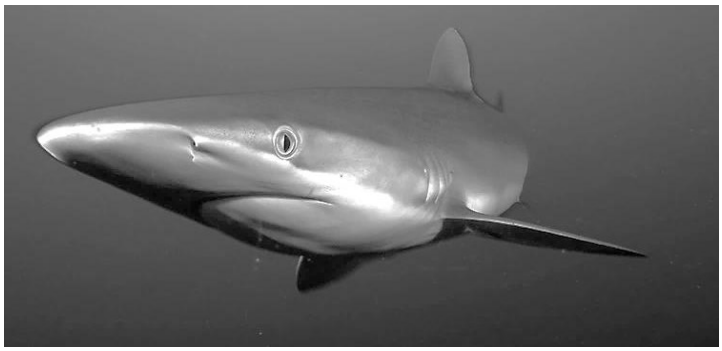

Figure 1.5: *Carcharhinus obscurus* shark.

Scalloped hammerhead sharks *Sphyrna lewini* (Figure 1.6), is a cosmopolitan shark species found in tropical and coastal warm waters. It can be found from the western Atlantic Ocean

to the Gulf of Mexico and the east coast of South Africa. These sharks are commonly caught in both pelagic long-line and coastal bottom long-line fisheries. *Sphyrna lewini* has a low fecundity and slow growth rate which causes it to have low population resilience against fishing pressures (de Bruyn *et al*. 2005; Piercy *et al*. 2007). Within the Tugela Bank off the central coast of KZN, *S. lewini* is the most copiously caught by-catch shark species within the commercial prawn fishery. This is due the fishing of large amounts of newborn *S. lewini* as by-catch (de Bruyn *et al*. 2005; Dudley & Simpfendorfer 2006). These sharks are also caught by commercial and artisanal fisheries in Mozambique, but whether stocks are shared with KZN is not yet known (de Bruyn *et al*. 2005). *Sphyrna lewini* are easily caught in large numbers due to their schooling nature which makes them vulnerable to being overfished. Another problem arises with the misidentification of these animals as the three most commonly fished hammerhead species (*S. zygaena*, *S. lewini* and *S. mokarran*) are difficult to distinguish from one another, especially when still young. This leads to very little species specific catch data being recorded (Abercrombie *et al*. 2005). *Sphyrna lewini* has been listed as endangered by the IUCN with an unknown population trend (Baum *et al*. 2007).

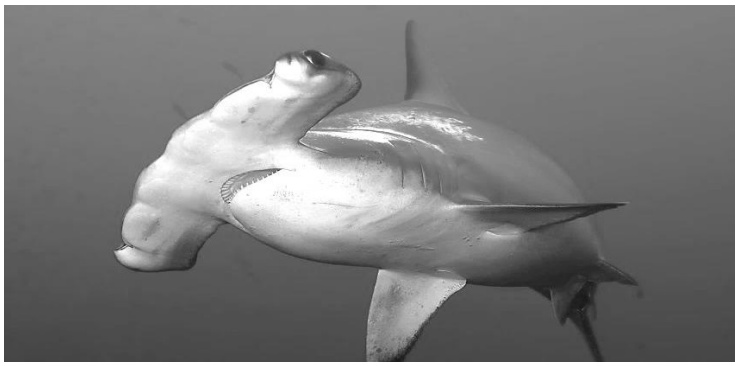

Figure 1.6: *Sphyrna lewini* shark.

1.5 Reproductive biology of shark species

The type of mating system that a species follows can have an impact on the species' genetic diversity (Griffiths *et al*. 2012). This in turn can influence adaptation to fluctuating environmental conditions and response to selection pressures (Daly-Engel *et al*. 2007). Increased vulnerability to population exhaustion and risk of extinction can be associated with a loss of genetic diversity. Studying the reproductive strategies of vulnerable marine species will allow conservation biologists to develop strategies to best conserve these animals. Sharks have many different methods of reproducing such as placental viviparity (live bearing

young), oviparity (egg producing) and aplacental viviparity. This makes them ideal for testing mating systems and sexual selection. Although the reproduction methods have been well studied, the prevalence of polyandry and multiple paternity in these species are not known (Byrne & Avise 2012).

Multiple paternity arises when multiple males fertilize a single brood of offspring (Daly-Engel *et al*. 2006, 2010). This can occur when female sharks engage in polyandry (Carrier *et al*. 1994), the act of multiple mating. Many taxa including amphibians, reptiles, mammals, insects, fish and crustaceans have shown multiple paternity to be a common strategy (Daly-Engel *et al*. 2010). Female sharks pose the ability to store viable sperm within the shell gland which could allow the sperm to mix and increase the potential of multiple sires (Daly-Engel *et al*. 2006; Byrne & Avise 2012). Multiple paternity has been reported in several orders such as Carcharhiniformes (Feldheim *et al*. 2004; Chapman *et al*. 2004; Daly-Engel *et al*. 2007), Orectolobiformes (Saville *et al*. 2002), Hexanchiformes (Larson *et al*. 2010), Squaliformes (Lage *et al*. 2008; Daly-Engel *et al*. 2010) and Lamniformes (Griffiths *et al*. 2012; Gubili *et al*. 2012).

The benefits of multiple mates are not yet known. Genetically, polyandry may lead to an increased chance in offspring survival, reduce the chances of inbreeding and increase the female's likelihood of mating with a male with genes more compatible to her own (Zeh & Zeh 1997, 2001; Madison *et al*. 2005; Neff & Pitcher 2005; Daly-Engel *et al*. 2007, 2010). On a larger scale, multiple paternity could increase the effective population size (due to more males successfully mating), thereby protecting the population against loss of allelic diversity (Daly-Engel *et al*. 2010). However, producing less offspring per male might also lead to a reduction in effective population size due to males siring less offspring than would have been possible in monogamous mating. The effective population size is reduced due to fewer offspring receiving the genes of a particular male which in turn means that fewer offspring will be able to pass on those same genes to the next generation once they sexually matured (Nunney 1993; Ramakrishnan *et al*. 2004; Karl 2008; Daly-Engel *et al*. 2010).

Fisheries can affect the dynamics of a population (size and age composition) in several ways. Firstly, fisheries have a direct effect on the mean size of the individual fish as well as population density. Secondly, density-dependent mechanisms and phenotypic plasticity are affected by fisheries, which in turn have an effect on growth as well as reproduction. Lastly, long term harvesting can mediate the drive of selective pressures within populations, which

again could lead to a shift in selective advantage (Rochet 1998). Whereas teleost fishes can respond more rapidly to fishing pressures, sharks are slower to compensate when population decline sets in (Musick *et al*. 2000). According to Worm *et al*. (2013) 48% of shark populations facing exploitation are being fished above their rate of rebound. By overfishing shark species it creates a shift in the species' abundance, which could alter population parameters, demographic characteristics (such as growth and reproduction patterns) and size structure at the species level as well as decrease population diversity (Rochet 1998; Stevens *et al*. 2000; Dulvy *et al*. 2013).

Many species have seen a gradual shift to small sizes. This in part could also be attributed to the fishing gears' size-selective properties (Bianchi *et al*. 2000; Stevens *et al*. 2000). Larger fish tend to be removed first by fisheries, followed by smaller and smaller fish being caught as stocks start to be depleted. Sharks such as Mustelus spp., *Sphyrna tiburo* and Rhizoprionodon spp., which tend to be smaller in size and mature quicker, tend to have higher rebound potential as opposed to species such as *S. lewini*, *C. obscurus* and *C. plumbeus* which are larger in size and have slower growth and maturing rates (Stevens *et al*. 2000; Dulvy *et al*. 2013). Greater reproductive potential is usually linked to populations with greater proportions of larger bodied fish. Therefore, by removing larger fish from the population, reproductive output within the population will decrease (Stevens *et al*. 2000).

Within mixed fisheries, sharks with lower rebound potential are driven to either stock collapse or extinction whereas the higher rebound potential sharks keep on supporting fisheries (Musick *et al*. 2000; Stevens *et al*. 2000). Fisheries also tend to fish in deeper waters once shallow water fish reserves have been exhausted. This in itself is problematic as deep-water Chondricthyan species are more susceptible to over-exploitation than Chondricthyans found in shallower waters as they have higher longevity, slower growth rates and mature at a later age in comparison to shallow water Chondricthyans (Garcia *et al*. 2008). Coastal species are also at risk due to a combination of fisheries and habitat destruction (Dulvy *et al*. 2013).

1.6 Molecular markers in conservation genetics

Within an aquatic environment direct observation of species migration patterns and behaviour is challenging. Fisheries management therefore needed an alternative method of evaluating stock structures and level of diversity within populations (O'Connell & Wright 1997).

Genetic variations within organisms are caused by mutations in their DNA, brought on by interactions with their environment as well as normal cellular metabolisms. Genetic drift and natural selection also play a key role in driving variation between species, populations and individuals (Lui & Cordes 2004). It is these genetic variations that allow scientists to investigate species on a genomic level in order to better understand population structure, population demography and diversity (Lui & Cordes 2004; Dudgeon *et al*. 2012). The use of molecular markers for assessing various population parameters often provide new insights into behaviour, responses to the environment, current stock delimitations and connectivity between sub-populations (Fromentin *et al*. 2009).

Allozymes were the first true molecular markers developed and were used in the study of elasmobranchs for the first time in 1986. The latter study focused on the *Mustelus lenticulatus* smoothhound and *Prionace glauca* blue sharks (Dudgeon *et al*. 2012). In 1980, Wyman and White discovered minisatellites (O'Connell & Wright 1997). These molecular markers enabled genetic identification, but could not be used for population genetics due to the complexity of the banding patterns they produced (O'Connell & Wright 1997; Schlötterer 2004). This was followed by the use of variable number tandem repeat loci (VNTRs) (O'Connell & Wright 1997; Wan *et al*. 2004). The mid 1990s saw the rise of a second wave of VNTRs, namely microsatellite markers, which widely became the marker of choice for application in management strategies (O'Connell & Wright 1997).

It was however the discovery of the polymerase chain reaction (PCR) by Kary Mullis that paved the way for the development of DNA-based molecular markers. It was no longer necessary to isolate extremely pure genomic DNA. Any genomic region could now be analysed after PCR amplification (Schlötterer 2004). Highly conserved regions were the first to be examined in early elasmobranch DNA studies which focused on phylogenetic analysis (Dudgeon *et al*. 2012). Microsatellite markers were the first markers to be widely used in conjunction with PCR amplification (Barbara *et al*. 2007). Microsatellite markers comprise of multiple copies of tandem organized simple sequence repeats (SSRs) and range in size from one to six base pairs. They tend to follow a stepwise mutation model as the mutation in the parental allele only differs by one or two repeats (Lui & Cordes 2004; Schlötterer 2004; Hoffman & Nichols 2011). These markers also have shorter repeat motifs in comparison to minisatellite markers and are evenly allotted throughout the genome (Schlötterer 2004; Wan *et al*. 2004). Furthermore, microsatellites are highly polymorphic which makes them extremely useful in population genetics, stock structure, paternity testing, genome mapping,

parentage, kinship and forensic studies (Lui & Cordes 2004; Schlötterer 2004; Dudgeon *et al*. 2012). Problems do however arise with scoring alleles due to PCR artefacts (such as stutter bands), presence of null alleles as well as complex mutation patterns which made population-genetic analysis difficult (Schlötterer 2004; Fromentin *et al*. 2009). Initially, microsatellite markers were isolated through the enrichment of genomic libraries for repetitive motifs. Plasmids were used to isolate positive clones and these clones were then sequenced and primers designed from the flanking regions. Polymorphism was finally evaluated through the amplification of the regions defined by the primers (Hoffman & Nichols 2011; Silva *et al*. 2013). Although this method of microsatellite isolation is a simple process, positive clone yields averaged between only 2-3%, making this method highly inefficient (Hoffman & Nichols 2011). Later methods included microsatellite-enriched genomic DNA libraries which were made in order to develop a higher number of potential markers (Liu & Cordes 2004; Silva *et al*. 2013) but even markers that did amplify successfully could turn out to be monomorphic. This is regularly observed in species that inherently have low genetic diversity levels (Hoffman & Nichols 2011).

Single nucleotide polymorphism can be described as a single base pair change brought on by a point mutation. This creates variations within an allele at a given locus and can be found every 0.3-1kb (Lui & Cordes 2004; Sprowles *et al*. 2006; Fromentin *et al*. 2009). DNA sequencing in 1977 allowed for the characterisation of these base pair substitutions, but it was not until the development of gene chip technology in the late 1990s that genotyping large amounts of SNPs were possible. Single nucleotide polymorphisms became one of the most promising markers as they could detect hidden polymorphism, were the most plentiful polymorphism throughout the genome (can be used in genome wide scans), can possess high information content, could be adapted to automation and can serve as powerful analytical tools for various genetic applications such as identification of candidate genes for QTL and genome mapping (Lui & Cordes 2004; Morin *et al*. 2004, 2009; Ryynanen *et al*. 2007).

In comparison to microsatellite loci and mtDNA, SNPs have a lower rate of mutation and variability (Schlötterer 2004; Fromentin *et al*. 2009; Morin *et al.* 2009), which eliminates the occurrence of homoplasy (Seddon *et al.* 2005), possess simple mutation models of evolution (Ryynanen *et al*. 2007), and are widespread and abundant in genomes (Fromentin *et al*. 2009; Morin *et al*. 2009). However, due to their bi-allelic nature, SNPs have low information content (Vignal *et al*. 2002; Schlötterer 2004). They are also expensive to isolate (Lui & Cordes 2004; Schlötterer 2004), have ascertainment bias (problem with measuring the true

frequency of the SNP) and possess a high rate of heterogeneity among sites (Schlötterer 2004). Several SNPs could be present at a single locus leading to the identification of thousands of genome-wide SNPs (Seddon *et al*. 2005). Furthermore, due to SNPs following a simpler mutation model, large scale genotyping can be done rapidly, cost-effectively and produce relatively low genotyping error rates. SNPs are also often linked to coding regions within the genome which can be used to distinguish functionally important polymorphisms more effectively (Ryynanen *et al*. 2007). It has also been proposed that digital DNA signatures could be constructed for animals using standardised SNP sets (Vignal *et al*. 2002). Several approaches for SNP identification exist. The most elementary strategy is to screen expressed sequence tags (ESTs) in order to identify polymorphic sites. A more robust method involves the pooling of individuals' DNA and sequencing it using shotgun genome sequencing (Lui & Cordes 2004; Schlötterer 2004). This however, produces copious amounts of data which can be difficult to analyse.

With the advancement of Next-Generation Sequencing (NGS) technologies, it has become possible to identify thousands of molecular markers such as microsatellites and SNPs. These platforms have made it possible to generate high genome coverage with increased throughput while reducing the cost of sequencing (Etter *et al*. 2011). Next-Generation Sequencing can be utilized as an alternative method to whole genome re-sequencing by rather focusing on compact panels of genomic markers throughout the genome. Ideally, platforms such as Illumina create large numbers of short reads. In order to avoid assaying orthologous regions, a large number of repeated reads need to be focused on the same genomic region. Genome sub-sampling can be attained by using a method called Restriction site Associated DNA sequencing (RAD sequencing), which creates short DNA fragments which are adjacent to recognition sites of specific restriction enzymes. Restriction site associated DNA sequencing is especially good to use when studying organisms lacking a reference genome as it can accelerate SNP discovery which in turn could be typed for hundreds or thousands of markers throughout the genome. This is accomplished by focusing on a subset of genomic regions from several individuals and sequencing it (Baird *et al*. 2008; Etter *et al*. 2011; Davey *et al*. 2013). SNPs are detected by creating a reduced version of the genome which allows the nucleotides next to the restriction enzyme sites to be over-sequenced. By selecting the correct restriction enzymes, the correct number of markers needed for an application can be chosen, whilst adding additional restriction enzymes will increase the number of markers that can be

identified. RAD sequencing can be used to genotype populations that have been pooled as well as the multiplex of individuals (Baird *et al*. 2008).

Restriction site associated DNA sequencing (Figure 1.7) entails the digestion of genomic DNA using restriction enzymes and ligation of adapters (P1) to the compatible ends. The ligated adapter consists of a barcode differing by three nucleotides (for identification purposes), forward amplification as well as primer sites. Fragments containing the adapters are then pooled and sheared at random, followed by an electrophoresis step in which fragments of a specific size are selected. A second adapter (P2) is then ligated onto the correct sized fragments. The reverse primer can only bind to the fragment when both the adapters (P1 and P2) are present – this is due to the Y shaped nature of the P2 adapter. The two strands are only complementary for part of the sequence due to the adapters' divergent ends. Thus only fragments ligated with the adapters will be amplified during the Polymerase chain reaction step (Baird *et al*. 2008; Etter *et al*. 2011).

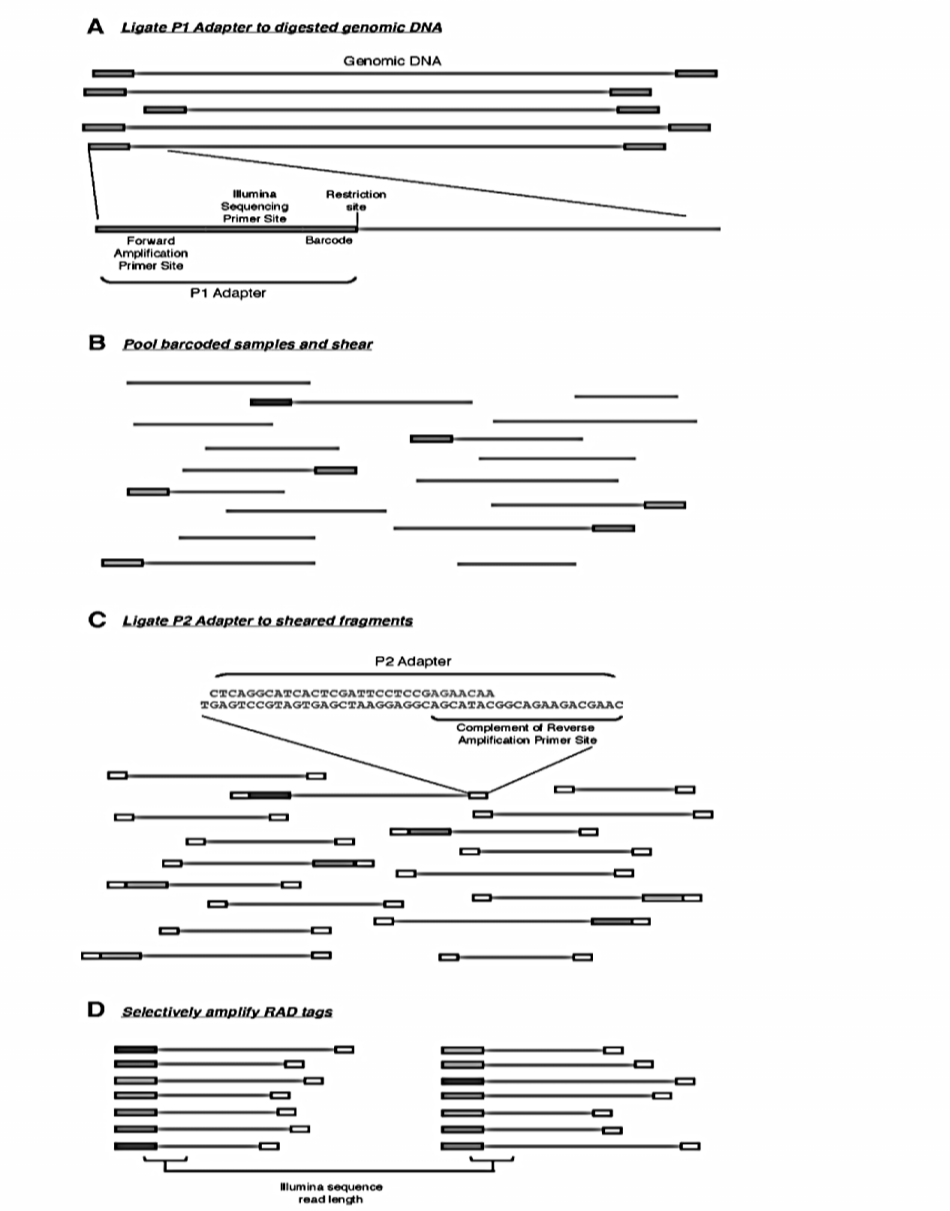

Figure 1.7: (A) P1 adapter ligated to digested genomic DNA. P1 adapter contains a barcode, forward primer set and primer site. (B) Fragments are pooled and randomly sheared. (C) P2 adapters, containing a reverse compliment of reverse amplification primer site, are ligated onto the fragments. (D) Enrichment of RAD tag containing both the P1 and P2 adapters (Baird *et al*. 2008).

Instead of using reads from the entire genome, which can be computationally straining, RAD sequencing produces short sheared-end sequencing which can then be compiled into larger contigs. This reduces the amount of sequencing errors when attempting to find noteworthy overlaps in the sequence. Thus multiplexing with RAD tag libraries and parallel sequencing can lead to the generation of vast amounts of polymorphism data (Etter *et al*. 2011; Rowe *et al*. 2011; Davey *et al*. 2013).

1.7 Research rationale and study outline

Since the development of genomic and genetic technology, the field of conservation genetics has increasingly been growing, taking advantage of techniques that use the variation within microsatellite and SNP markers as the predominant tools of study (DeSalle & Amato 2004). Concepts within conservation genetics have made it possible to identify, describe and understand the motifs and mechanisms that lead to the endangerment of a population or species. Comparable measurements such as gene flow, genetic variation, inbreeding as well as minimum viable and effective population size, help quantify the mechanisms that lead to the endangerment of populations (DeSalle & Amato 2004; Wan *et al*. 2004).

Sharks have inhabited our oceans for millions of years. They are however vulnerable to excessive exploitation by fisheries due to their slow growth rates and reaching sexual maturity at a later age than teleost fishes. Not much research has been conducted on the genetic diversity and population connectivity of these animals which limits management and biodiversity conservation of these species. Prior studies have focussed on developing species-specific microsatellite markers or cross-amplifying already developed markers to closely related taxa. The development of species-specific microsatellite and SNP markers within shark species will contribute to the growing amount of data which can be used to genotype individuals, analyse the underlying population structure of a species, aid in species identification and establish genetic parameters within a species which in turn could contribute to the establishment of improved management and conservation strategies. This study also investigated the presence of multiple paternity within these shark species as long-term conservation strategies will also require knowledge on shark behavior and demographics such as mating systems. This study therefore aimed to develop standardized species-specific marker panels with which the presence of multiple paternity could be assessed as well as to identify species-specific microsatellite and SNP sites through Next-Generation Sequencing.

1.8 Study outline

This study was divided into two parts which will contribute not only to the growing genetic resources for sharks but also add to existing knowledge on mating behaviour within the order Carcharhiniformes. Firstly, this study investigated the presence of multiple paternity within *Mustelus mustelus*, *Carcharhinus obscurus* as well as *Sphyrna lewini* (Chapter 2). Secondly, this study attempted to identify species-specific microsatellite and SNP markers for *Mustelus mustelus* and *Carcharhinus obscurus* (Chapter 3). The first experimental chapter (Chapter 2)

entailed the extraction of genomic DNA from several *M. mustelus*, *C. obscurus* as well as *S. lewini* litters (pups and mothers). Previously developed microsatellite loci were then selected for the testing of multiple paternity through parentage analysis. A standardised marker panel was then constructed for each species based on preliminary test results. These marker panels were then further tested on additional litters for each species to determine whether marker panels could be used as standardised panels to test for multiple paternity within these species. This chapter has already been submitted to Journal of Fish Biology for publication and is currently under review (JFB MS 15-334). The second experimental chapter (Chapter 3) of this study entailed acquiring samples and extracting DNA from *M. mustelus* and *C. obscurus*, followed by reduced genome sequencing through the use of Next-Generation Sequencing technology. This was followed by bioinformatic analysis of both sequencing data sets for potential microsatellite and SNP marker discovery. This chapter is intended for publication in Conservation Genetic Resources reporting on the development and characterization of the first species-specific markers for *M. mustelus* and *C. obscurus*.

All of the above will increase our growing genetic data resources available for these animals. The identification of the species-specific markers will allow for more effective species identification, population structure analysis, and a better understanding of demographic parameters that govern a species' susceptibility to overfishing. By incorporating information generated from the MP assessment as well as future genetic diversity and population structure analysis, improved conservation and management programs can be put in place to better conserve biodiversity within these shark species.

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CHAPTER 2

Assessing multiple paternity in three commercially exploited shark species occurring in southern Africa: *Mustelus mustelus*, *Carcharhinus obscurus* and *Sphyrna lewini*

ABSTRACT

To date, polyandry and multiple paternity (MP) have been reported in several elasmobranch species. In this study, MP was investigated in three commercially important shark species, common smoothhound *Mustelus mustelus*, dusky shark *Carcharhinus obscurus* and scalloped hammerhead *Sphyrna lewini*. Prior to analysis, 22 microsatellite markers were tested for their ability to detect MP in a subset of the *C. obscurus* and *S. lewini* litters. Reduced marker panels of between five and six microsatellites were constructed based on their polymorphic information content and ability to be multiplexed. The reduced marker panels were used to genotype a total of 204 individuals and assess the presence of multiple paternity in six litters of *M. mustelus,* ten litters of *C. obscurus* (total of fourteen) and nine litters of *S. lewini* (total of thirteen). Analysis in GERUD and COLONY revealed the presence of MP in all three species. Multiple paternity was observed in 67%, 35% and 46% of the litters of *M. mustelus*, *C. obscurus* and *S. lewini*, respectively; with corresponding average number of sires 1.6, 1.4 and 2.0. The variation in the rate of MP among the three species is not unexpected while the comparatively high frequency of MP observed for *M. mustelus*, matches what has previously been reported in shark species demonstrating aggregation behaviour.

2.1 INTRODUCTION

2.1.1 Fishing concern for Chondrichthyans

Directed fisheries have been a well-documented cause of elasmobranch population collapse (Daly-Engel *et al*. 2006) which has raised questions on whether large scale shark fisheries are sustainable. In comparison to teleost fish, sharks are less fertile, reach sexual maturity at a later age, produce less young and have a slow growth rate which makes them vulnerable to overfishing (Musick *et al*. 2000; Stevens *et al*. 2000; Daly-Engel *et al*. 2010; Byrne & Avise 2012; Worm *et al*. 2013). Due to their K selected life strategies, any loss of genetic diversity could increase a species' vulnerability to population exhaustion and risk of extinction. Furthermore, an increase in the demand for shark related products coupled with their inherent vulnerability has led to the continuous depletion of shark populations world-wide (Byrne & Avise 2012). Several factors regarding shark ecology should be taken into account when designing effective long-term conservation strategies. Apart from assessing stock structure and life history traits, it has become apparent that data on mating systems are paramount in order to monitor shark populations more accurately (Neff & Pitcher 2002; Chapman *et al*. 2004). The type of mating system can impact a species' genetic diversity which in turn can influence adaptation to fluctuating environmental conditions and response to selection pressures (Daly-Engel *et al*. 2007; Griffiths *et al*. 2012).

2.1.2 Modes of reproduction and the presence of multiple paternity

Different modes of reproduction such as placental or aplacental viviparity and oviparity are exhibited by shark species which make them ideal for testing mating systems and sexual selection. Although these methods of reproducing have been well studied, the prevalence of polyandry and multiple paternity (MP) in these species are not well known (Neff & Pitcher 2002; Byrne & Avise 2012; Boomer *et al*. 2013). Multiple paternity can be defined as

multiple males fertilising a single brood of offspring. These multiple matings are referred to as polyandry (Daly-Engel *et al*. 2006) and have been reported in several elasmobranch species, including placental and aplacental live bearers as well as oviparous sharks and rays (Boomer *et al*. 2013). Through the use of molecular markers, MP has been identified in several orders of sharks such as Carcharhiniformes, Hexanchiformes, Lamniformes, Orectolobiformes and Squaliformes. Most MP studies have focused on shark species with a viviparous reproductive strategy as these litters can be easily identified. To date, MP has been genetically identified in 17 shark and one ray species using as little as four microsatellite loci (Table 2.1 summarizes all published multiple paternity studies to date). Most studies have focused on assessing the presence of multiple paternity within schooling sharks (for example Tope shark *Galeorhinus galeus*, the Rig shark *Mustelus lenticulatus* and the Sandbar shark *Carcharhinus plumbeus*) rather than non-schooling sharks such as the Whale shark *Rhincodon typus*. This might be due to non-schooling shark species being harder to obtain for sampling.

Table 2.1: Multiple paternity studies to date including species studied, mode of reproduction, number of litters per study and number of microsatellite markers used for analysis.

Family	Species	Number of litters	Average number of offspring	Average number of sires	Mode of reproduction	Number of microsatellites	% of litters with MP	References
Carcharhinidae	***Carcharhinus altimus*** Bignose shark	1	9	1	viviparous	8	NA	Daly-Engel *et al*. 2006
	Carcharhinus galapogensis Galapogos shark	1	7	1	viviparous	8	NA	Daly-Engel *et al*. 2006
	Carcharhinus plumbeus Sandbar shark	1	7	1	viviparous	8	NA	Daly-Engel *et al*. 2006
	Carcharhinus plumbeus Sandbar shark	20	9.4	2.3	viviparous	5	85	Portnoy *et al*. 2007
	Carcharhinus plumbeus Sandbar shark	20	5.5	1.4	viviparous	6	40	Daly-Engel *et al*. 2006
	Negaprion brevirostris Lemon shark	1	11	1	viviparous	3	NA	Feldheim *et al*. 2001
	Negaprion brevirostris Lemon shark	97	6.7	2	viviparous	9	87	Feldheim *et al*. 2004
	Negaprion brevirostris Lemon shark	85	4.3	2	viviparous	11	81	DiBattista *et al*. 2008
Scyliorhinidae	***Scyliorhinus canicula*** Small-spotted cat shark	13	11.5	2.4	oviparous	12	92	Griffiths *et al*. 2012

Sphyrnidae	***Sphyrna tiburo*** Bonnethead shark	22	8.5	1.2	viviparous	4	19	Chapman *et al*. 2004
Triakidae	***Galeorhinus galeus*** Tope shark	5	30	1.5	ovo-viviparous	6	40	Hernandez *et al*. 2014
	Mustelus antarcticus Gummy shark	29	11.3	1.4	aplacental ovo-viviparous	8	13	Boomer *et al*. 2013
	Mustelus asterias Starry smoothhound shark	12	9.6	1.6	aplacental ovo-viviparous	4	58	Farrell *et al*. 2014
	Mustelus henlei Brown smoothhound	14	13.2	2.3	placental viviparous	4	93	Byrne & Avise 2012
	Mustelus henlei Brown smoothhound	18	10	1.4	placental viviparous	4	40	Chabot & Haggin 2014
	Mustelus lenticulatus Rig shark	19	4	1.5	aplacental viviparous	8	24	Boomer *et al*. 2013
	Mustelus mustelus Common smoothhound	19	11	2.0	viviparous	9	47	Marino *et al*. 2015
	Mustelus punctulatus Black-spotted smoothhound	13	23	2.1	viviparous	9	54	Marino *et al*. 2015
Ginglymostomatidae	***Ginglymostoma cirratum*** Nurse shark	1	32	1	ovo-viviparous	NA	NA	Saville *et al*. 2002
Rhincodontidae	***Rhincodon typus*** Whale shark	1	29	1	ovo-viviparous	9	NA	Schmid *et al*. 2010
Rajidae	***Raja clavata*** Thornback ray	4	43.3	4.5	ovo-viviparous	5	100	Chevolot *et al*. 2007
Squalidae	***Squalus acanthias*** Spiny dogfish	10	5	1.3	aplacental viviparous	7	30	Lage *et al*. 2008
	Squalus acanthias Spiny dogfish	29	5.4	1.2	aplacental viviparous	7	17	Verissimo *et al*. 2011
	Squalus mitsukurii Shortspine spurdog	27	6.6	1.1	ovo-viviparous	8	11	Daly-Engel *et al*. 2010

2.1.3 Study species

The common smoothhound *Mustelus mustelus* (Linnaeus 1758) is a small, viviparous bottom-living shark (Filiz 2009; Zaera & Johnson 2011) found in temperate, continental shelf waters (Ebert *et al*. 2013). Regionally, they range from Angola and Namibia to KwaZulu-Natal (KZN) in South Africa (Compagno 1984; Compagno *et al*. 1989). These sharks are valued for their white meat and are caught by commercial and recreational line-fisheries, demersal shark

long-lines, small pelagic fisheries, inshore and offshore demersal trawl fisheries as well as hake long-line fisheries within South African waters (DAFF 2014). These sharks have been found to aggregate in large groups which lead to large quantities of *M. mustelus* being caught (Smale & Compagno 1997; Baum *et al*. 2007). Large quantities of these sharks being captured coupled with their population segregation, low fecundity and slow growth rate make them vulnerable to stock depletion (Baum *et al*. 2007; Piercy *et al*. 2007).

Dusky sharks *Carcharhinus obscurus* (Lesueur 1818) are large, viviparous apex predators from the Carcharhinidae family. They occur in tropical and warm-temperate shelf waters (Ebert *et al*. 2013) and are regionally found in the Red Sea, Mozambique, Madagascar and in South Africa from False Bay to KZN (Garrick 1982; Compagno 1984; Compagno *et al*. 1989). Their meat and skin are used for human consumption (Vannuccini 1999) and leather products respectively and vitamins are extracted from their liver oil (Musick *et al*. 2009). Within South African waters these sharks are caught by commercial line-fisheries, demersal and pelagic shark long-lines, hake long-lines fisheries, gill and beach seine net fisheries as well as bather protection gear in KZN (DAFF 2014). Due to their relatively slow growth rate, late maturation (approximately 20 years of age) and long gestation periods (possibly 2 years) (Natanson & Kohler 1996; Dudley *et al*. 2005) *C. obscurus* are potentially also vulnerable to overfishing (Hussey *et al*. 2009; Musick *et al*. 2009).

Scalloped hammerheads *Sphyrna lewini* (Griffith & Smith 1834) are viviparous cosmopolitan sharks that occupy tropical, warm-temperate coastal, pelagic and semi-oceanic waters (Ebert *et al*. 2013). Their range extends from the Red Sea to Mozambique and the east coast of South Africa (Compagno 1984; Compagno *et al*. 1989). Hammerhead sharks are the preferred export for Africa due to its high quality meat (Vannuccini 1999). Due to a global concern over the decline of this commercially exploited species, *S. lewini* was listed on the CITES Appendix II in 2013 (Convention on International Trade in Endangered Species of

Wild Fauna and Flora (CITES) 2015). Within South African fisheries, these sharks are caught in commercial line-fisheries, pelagic shark long-lines, tuna and swordfish pelagic long-lines, small pelagic fisheries, hake long-line fisheries as well as in the KZN bather protection gear (DAFF 2014).

2.1.4 Study rationale

There is still no global action plan to ensure proper implementation of management and conservation mechanisms after 20 years of creating awareness of Chondricthyan populations decline (Dulvy *et al*. 2013). Several countries, including South Africa have, however, implemented national conservation and management plans. Within the South African National Plan of Action for the Conservation and Management of Sharks (SA NPOA-Sharks), sharks are managed in terms of 1) the Dumping at Sea Control Act (1980), Marine Living Resources Act (1998), 2) the National Environmental Management: Protected Areas Act (2003), 3) the National Environmental Management: Biodiversity Act (2004) as well as 4) the KwaZulu-Natal Sharks Board Act (2008) (DAFF 2014). Several studies have stressed the importance of studying reproductive strategies in shark species in order to better assess these shark populations. At the time this study was embarked, no studies have yet reported on the presence or absence of MP in the three locally exploited shark species *M. mustelus*, *C. obscurus* or *S. lewini*. A very recent study by Marino *et al*. (2015) however, reported on the assessment of two Mustelus species, *M. mustelus* and *M. punctulatus*, from the northern Adriatic Sea. Assessment confirmed the presence of MP in both species. The current study aimed to 1) optimise microsatellite marker panels for use in MP assessment and 2) infer MP in three commercially exploited species using these marker panels.

2.2 MATERIALS AND METHODS

2.2.1 Sampling and DNA extraction

Fin clip and muscle tissue from six litters ranging from five to 18 pups for *M. mustelus*, 14 litters ranging from three to 14 pups for *C. obscurus* and 13 litters ranging from three to 16 pups for *S. lewini* were obtained from the east coast of South Africa. All samples were taken from sharks caught in the bather protection program off KZN (Figure 2.1). For details of the program see Dudley *et al*. (2005). Fin clips were preserved in 99,9% ethanol and total DNA was extracted using a modified CTAB protocol of Saghai-Maroof *et al*. (1984). The concentration of every buffer reagent was doubled and the incubation time extended from 60 minutes to overnight. Instead of Chloroform: octanol (24:1), Chloroform: isoamylalchohol (24:1) was used to separate DNA from cell tissue. Two volumes of 100% ethanol were added to precipitate DNA. Samples were rehydrated using MilliQ water instead of 1.5 mL NH_4OAc and 0.25 mM EDTA.

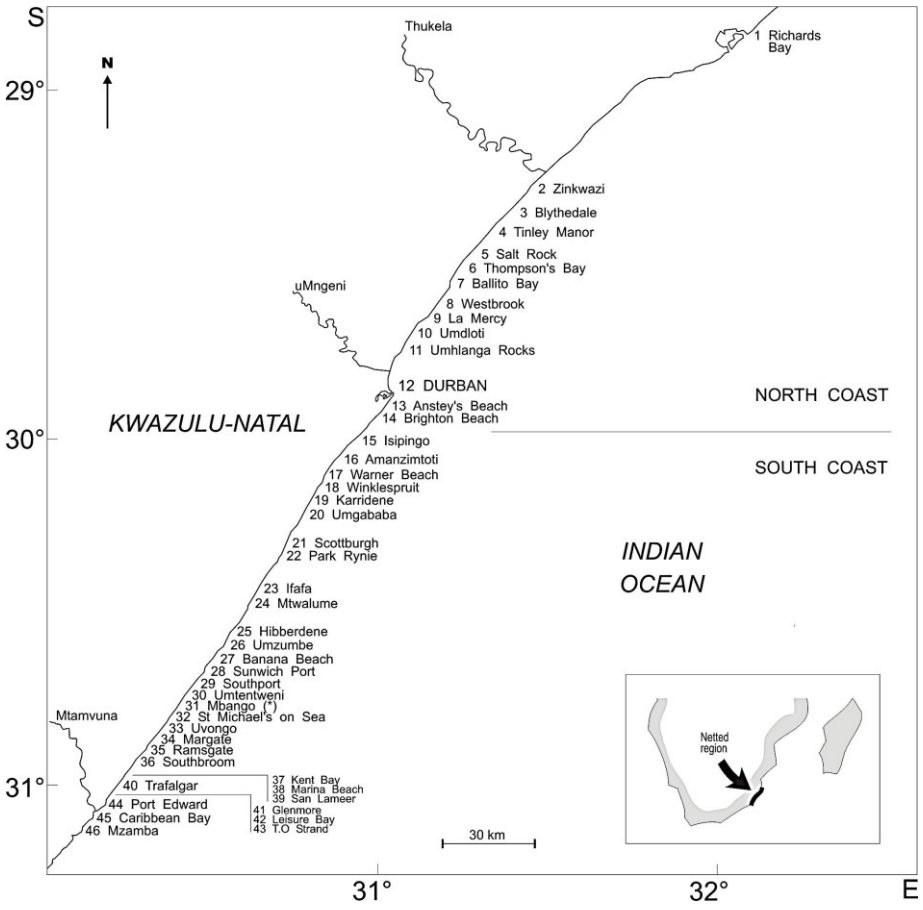

Figure 2.1: Locations of all bather protection nets along the coast of KwaZulu-Natal, South Africa. Samples were obtained from Amazimtoti (16), Ballito Bay (7), Blythedale (3), Durban (12), Kent Beach (37), Margate (34), Park Rynie (22), Port Edward (44) and Richards Bay (1).

2.2.2 Marker panel optimization

A total of 22 microsatellite markers previously developed for *M. canis* (Giresi *et al*. 2012), *M. henlei* (Byrne & Avise 2012; Chabot 2012) and *G. galeus* (Chabot & Nigenda 2011) were selected to test for their utility in assessing MP through PCR cross-amplification. Primers for microsatellite loci fluorescently labelled with FAM, VIC, PET or NED dyes (Life technologies), were grouped into four multiplex panels (Table 2.2). Initial testing of the multiplexes was conducted on four litters from *C. obscurus* and *S. lewini* each. Once the presence of MP was confirmed, the 22 microsatellite markers were reduced to a panel of five to six microsatellite markers per species. Basic genetic diversity estimates were used to select

the most informative microsatellite markers in order to create a unique marker panel for each species. For *M. mustelus*, a study by Maduna *et al*. (2014) was consulted in order to obtain basic diversity estimates for this species as samples for *M. mustelus* had not yet been received when initial marker testing was done.

Table 2.2: Twenty-two fluorescently labelled microsatellite markers grouped into four multiplex systems used to assess multiple paternity (MP) in *C. obscurus* and *S. lewini* prior to marker panel reduction. The fluorescent dye for each marker is in brackets.

Multiplex 1	Multiplex 2	Multiplex 3	Multiplex 4
Mh1 (VIC)	*McaB5* (VIC)	*Gg2* (NED)	*Gg15* (FAM)
Mh2 (VIC)	*McaB6* (FAM)	*Gg3* (PET)	*Gg17* (PET)
Mh9 (FAM)	*McaB22* (NED)	*Gg7* (NED)	*Gg18* (VIC)
Mh25 (FAM)	*McaB27* (PET)	*Gg11* (NED)	*Gg22* (FAM)
Mca25 (PET)	*Mca33* (FAM)	*Gg12* (FAM)	*Gg23* (VIC)
McaB39 (NED)	*McaB37* (NED)		

Multiplex PCR for the amplification of the selected loci was performed using a Qiagen Multiplex PCR kit (Qiagen). A 10µL reaction mixture was prepared, containing 50µg/µL DNA, 5x QM-Mix and 1x Primer mix (P_R-Mix) (prepared according to the manufactures protocol). PCR cycling conditions for Multiplex 1, 3 and 4 included an initial activation step of one cycle at 95°C for 15 minutes followed by 35 cycles of a denaturing step at 94°C for 30 seconds, annealing step at 59°C for 90 seconds and extension step at 72°C for 1 minute. A final extension step at 60°C for 30 minutes was conducted. PCR cycling conditions for Multiplex 2 included an initial activation step of one cycle at 95°C for 15 minutes followed by 35 cycles of a denaturing step at 94°C for 30 seconds, annealing step at 56°C for 90

seconds and extension step at 72°C for 1 minute. A final extension step at 60°C for 30 minutes was conducted. Product analysis was conducted on an ABI 3730XL DNA Analyzer (Life Technologies) with the LIZ®600 size standard and visualised using ABI PRISM GeneMapper Software 3.0 (Life technologies).

2.2.3 Data analysis

All loci were tested for genotyping errors such as presence of null alleles, stuttering and large allele dropout in MICROCHECKER 2.2.3 (Van Oosterhout *et al*. 2004). GENALEX (Peakall & Smouse 2012) and MICROSATOOLS (Minch *et al*. 1996) were used to calculate expected and observed heterozygosity as well as polymorphic information content. The software program GERUD 2.0 (Jones 2005) was used to estimate the minimum number of fathers for each litter. The program PRDM (Neff & Pitcher 2002) was used to calculate the probability of detecting MP. Four different scenarios were run for the PRDM software: (1) two sires with equal contributions (0,5: 0,5), (2) two sires with moderately skewed contributions (0,7: 0,3); (3) two sires with highly skewed contributions (0,9: 0,1) and (4) paternal skews as calculated in COLONY 2.0 (Jones & Wang 2010). Sibling relationships, as well as the most likely number of fathers were estimated using COLONY. Once the initial tests had confirmed MP in each of the two species, a reduced marker panel (shown in Table 2.3), was constructed for each of the species. Note that multiplex conditions for each of the multiplexes (provided in Table 2.3) refers back to the conditions of the original 22 microsatellite marker panel (Table 2.2). Thus for Multiplex A, the same PCR conditions that were used for Multiplex 2 will apply. The most polymorphic microsatellite markers from the Maduna *et al*. (2014) study were selected for the *M. mustelus* MP marker panel. This marker panel was then used to conduct MP analysis on the remainder of the litters from all three species.

Table 2.3: Reduced labelled microsatellite marker panels grouped into multiplexes for each species tested. Marker panel for *M. mustelus* consists of Multiplex A and B, marker panel for *S. lewini* consists of Multiplex C and D and marker panel for *C. obscurus* consists of Multiplex E.

Species	***Mustelus mustelus***		***Sphyrna lewini***		***Carcharhinus obscurus***	
Multiplex	Multiplex A	Multiplex B	Multiplex C	Multiplex D	Multiplex E	
Multiplex conditions	MP 2	MP 3 & 4	MP 2	MP 3	MP 1, 3 & 4	
	McaB5	*Gg2*	*McaB5*	*Gg2*	*Mh1*	*Gg11*
	McaB6	*Gg22*	*McaB22*	*Gg7*	*Mh25*	*Gg23*
	McaB22	*Gg23*	*McaB37*		*Gg3*	

2.3 RESULTS

Six litters of *M. mustelus*, fourteen litters of *C. obscurus* and thirteen litters of *S. lewini* were tested for the presence of multiple paternity (MP). Table 2.4 shows each litter tested as well as the number of pups per litter. Four litters of *C. obscurus* and four litters of *S. lewini* were used in the initial assessment of MP in order to optimize microsatellite marker panels for each species respectively. Table 2.5 shows amplification success across all 22 microsatellite markers for *C. obscurus* and *S. lewini*. Of the 22 markers tested, 50% of them amplified successfully for *C. obscurus* and 68% of the markers showed successful amplification in *S. lewini*.

Table 2.4: A summary of the litters of *M. mustelus*, *C. obscurus* and *S. lewini* assessed for presence of MP, indicating the number of pups per litter.

M. mustelus		***C. obscurus***		***S. lewini***	
Litter assessed	**Number of pups**	**Litter assessed**	**Number of pups**	**Litter assessed**	**Number of pups**
MA	15	**COA**	12	**SLA**	16
MB	17	**COB**	6	**SLB**	19
MC	13	**COC**	10	**SLC**	11
MD	10	**COD**	11	**SLD**	9
ME	5	**COE**	12	**SLE**	5
MF	7	**COF**	8	**SLF**	7
		COG	7	**SLG**	15
		COH	9	**SLH**	4
		COI	10	**SLI**	8
		COJ	16	**SLJ**	6
		COK	14	**SLK**	11
		COL	10	**SLL**	8
		COM	12	**SLM**	3
		CON	11		
Average litter size	**11**		**11**		**9**

Table 2.5: Amplification success of microsatellite markers tested for *C. obscurus* and *S. lewini*. (+) successful amplification; (-) unsuccessful amplification of marker.

Microsatellite marker	*Carcharhinus obscurus*	*Sphyrna lewini*
Mh1	+	+
Mh2	-	+
Mh9	-	-
Mh25	+	+
Mca25	+	-
McaB39	+	-
McaB5	-	+
McaB6	-	+
McaB22	+	+
McaB27	-	-
Mca33	-	-
McaB37	-	+
Gg2	-	+
Gg3	+	+
Gg7	-	+
Gg11	+	-
Gg12	-	-
Gg15	+	+
Gg17	+	+
Gg18	+	+
Gg22	-	+
Gg23	+	+
Total amplified	11/22	15/22

Polymorphic information content across the 18 loci ranged from 0.431 – 0.865 in *M. mustelus*, 0.127 – 0.682 in *C. obscurus* and 0.248 – 0.900 in *S. lewini* (See Table 2.6 for full diversity estimates). Basic diversity estimates for *M. mustelus* was obtained from Maduna *et al*. (2014) as these samples were sampled from different locations around the South African coastline and were therefore a good representation of the allelic richness for this species.

Table 2.6: Basic diversity estimates for *M. mustelus*, *C. obscurus* and *S. lewini* as calculated in GENALEX and MICROSATOOLS. H_e: Expected heterozygosity, H_o: Observed Heterozygosity and PIC: Polymorphic information content.

	Mustelus mustelus			*Carcharhinus obscurus*			*Sphyrna lewini*		
Marker	**H_e**	**H_o**	**PIC**	**H_e**	**H_o**	**PIC**	**H_e**	**H_o**	**PIC**
Mh1	0.544	0.885	0.443	0.146	0.153	0.127			
Mh25				0.770	0.888	0.682			
McaB5	0.716	0.826	0.674	0.516	0.702	0.439	0.588	0.467	0.476
McaB6	0.702	0.756	0.655						
Mca25				0.333	0.368	0.279			
Mca33	0.674	0.872	0.609						
McaB22	0.882	0.874	0.865	0.341	0.382	0.290	0.698	0.618	0.904
McaB37	0.486	0.483	0.431				0.697	0.605	0.808
McaB39				0.447	0.528	0.374			
Gg2	0.688	1.000	0.632				0.225	0.185	0.248
Gg3				0.576	0.772	0.441	0.387	0.337	0.388
Gg7							0.729	0.642	0.900
Gg11				0.719	0.913	0.620			
Gg15							0.737	0.645	0.759
Gg17							0.228	0.178	0.229
Gg18	0.558	0.976	0.456				0.745	0.654	0.722
Gg22	0.559	0.964	0.455						
Gg23	0.651	1.000	0.582	0.468	0.675	0.347	0.363	0.298	0.283

Polymorphic information content was considered in order to reduce the marker panels to only the most informative markers (Table 2.3). As these markers were not species specific but cross-amplified from other closely related species, only markers which showed little or no presence of null alleles and moderate to high polymorphic information content were selected for MP assessment in the rest of the litters. Cross-amplification using reduced marker panels was successful across all three species. Multiple paternity was detected within all three species using a maximum of six markers for *M. mustelus* and a minimum of five markers for *C. obscurus* and *S. lewini*, respectively. Litter sizes, results of GERUD and COLONY software as well as PRDM results calculated using both paternal contributions as calculated in GERUD and COLONY are summarised in Table 2.7. *Mustelus mustelus* had an average of 10 pups per litter with an average of 1.6 sires assigned to each litter estimated by GERUD software (see Table 2.7 for number of sires per litter). PRDM was highest with a 0.5:0.5 skew with an average of 44,9% detection. *Carcharhinus obscurus* litter size averaged at eight pups per litter and yielded an average of 1.4 sires estimated with GERUD software (see Table 2.7 for number of sires per litter). The average probability of detected MP (PRDM) was 26,1% (0.5:0.5 skew). *Sphyrna lewini* had an average litter size of seven pups and an average of 2.0 sires per litter as calculated in GERUD software (see Table 2.7 for number of sires per litter). PRDM was highest with a 0.5:0.5 skew with an average of 43,2% detection. Overall, MP was detected in 67% of *M. mustelus*, 35% of *C. obscurus*, and 46% of *S. lewini* litters tested (see Table 2.7 for number of sires per litter).

Table 2.7: A summary of multiple paternity (MP) assessment in shark litters including GERUD minimal analysis results with equal, moderate and highly skewed contributions, COLONY maximum likelihood results with paternal skew and probability of detecting MP calculated in PRDM. MM- abbreviated litters represent *M. mustelus*, CO-abbreviated litters represent *C. obscurus* and SL- abbreviated litters represent *S. lewini*.

			GERUD				COLONY	
			Paternal skew used in PRDM					
Sample	Litter size	No. sires	0.5 : 0.5	0.7 : 0.3	0.9 : 0.1	No. sires	Paternal skew	PRDM
MMA	12	2	0.782	0.750	0.488	3	5:3:4	0.937
MMB	13	2	0.494	0.466	0.285	6	2:2:4:1:2:2	0.896
MMC	14	2	0.695	0.650	0.394	4	5:6:2:1	0.896
MMD	9	1	0.279	0.248	0.125	3	3:3:3	0.437
MME	5	1	0.203	0.169	0.075	3	1:2:2	0.285
MMF	7	2	0.239	0.204	0.093	3	5:1:1	0.362
Average	**10**	**1.6**	**0.449**	**0.415**	**0.243**	**3.6**	**-**	**0.634**
COA	8	2	0.537	0.473	0.234	5	1:1:1:3:2	0.925
COB	3	1	0.068	0.057	0.025	2	1:2	0.297
COC	6	1	NA	NA	NA	1	3:2:1	NA
COD	7	2	0.495	0.434	0.207	4	1:3:1:2	0.794
COE	11	1	0.276	0.249	0.129	2	4:7	0.266
COF	7	1	0.112	0.102	0.047	3	1: 2: 4	0.153
COG	7	1	0.161	0.144	0.064	2	5: 2	0.135
COH	10	1	0.189	0.172	0.098	7	2:2:1:1:1:2:1	0.504
COI	6	2	0.395	0.348	0.174	5	1:2:1:1:1	0.697
COJ	7	1	0.000	0.000	0.000	2	4:3	0.000
COK	12	2	0.456	0.422	0.241	3	5:4:3	0.669
COL	14	1	0.232	0.214	0.120	4	1:10:2:1	0.313
COM	5	1	0.222	0.193	0.093	2	4:1	0.153
CON	11	2	0.249	0.240	0.135	3	6:3:2	0.383
Average	**8**	**1.4**	**0.261**	**0.234**	**0.121**	**3.2**	**-**	**0.406**

SLA	10	3	0.801	0.743	0.421	6	2:1:2:1:2:2	0.999
SLB	16	4	0.874	0.840	0.582	8	1:2:6:1:1:2:2:1	0.999
SLC	4	2	0.337	0.283	0.123	3	2:1:1	0.894
SLD	8	5	0.719	0.645	0.339	6	2:1:1:2:1:1	0.979
SLE	5	1	0.088	0.076	0.033	3	1:2:2	0.131
SLF	6	1	NA	NA	NA	1	NA	0.000
SLG	7	3	0.718	0.677	0.414	5	1:2:1:2:1	0.963
SLH	4	1	NA	NA	NA	1	4	0.000
SLI	6	1	0.205	0.179	0.085	4	1:3:1:1	0.345
SLJ	5	1	0.160	0.136	0.061	3	1:1:3	0.202
SLK	3	2	0.039	0.033	0.014	3	1:1:1	0.056
SLL	7	1	0.388	0.346	0.170	5	1:2:1:2:1	0.679
SLM	6	1	0.419	0.372	0.188	3	4:1:1	0.480
Average	**7**	**2**	**0.432**	**0.394**	**0.221**	**4**	**-**	**0.517**

2.4 DISCUSSION

Multiple paternity was identified in all three shark species (67% of *M. mustelus*, 35% of *C. obscurus* and 46% of *S. lewini* litters) using the reduced microsatellite marker panels optimised for each species. Although comparable with that previously reported for similar species, this could be a conservative estimation, as spontaneous abortion can occur during capture and/or a small percentage of retrieved pups were not genotyped successfully. *Mustelus mustelus* showed a relatively high occurrence of MP (67%), which is comparable to that of the Adriatic *M. mustelus* (47%) (Marino *et al*. 2015) and other *Mustelus* species such as, *M. punctulatus* (54%) (Marino *et al*. 2015), *M. asterias* (58%) (Farrell *et al*. 2014) and *M. henlei* (40-93%) (Byrne & Avise 2012; Chabot & Haggin 2014). When comparing species with placental modes of reproduction to that of non-placental modes of reproduction it has

previously been questioned whether placental shark species (e.g. *M. henlei*) have a higher occurrence of MP in comparison to non-placental (*M. antarcticus* and *M. lenticulatus*) shark species (Boomer *et al*. 2013). Given the fact that *M. asterias* is non-placental and has a MP rate of > 50%, and also most similar to that reported for *M. mustelus* here, it supports the notion that MP frequency in *Mustelus* is not necessarily a consequence of differential reproductive mode alone.

Carcharhinus obscurus from South Africa is now the second Carcharhinus species worldwide in which MP has been confirmed and it's frequency of MP is similar to that of. *C. plumbeus* (40%) investigated in Hawaii (Daly-Engel *et al*. 2006) and also compares to other species such as *G. galeus* (40%) (Hernandez *et al*. 2014). *Sphyrna lewini* also showed a much higher percentage of MP (46%) in comparison to the only other hammerhead shark species investigated, *S. tiburo* (19%) (Chapman *et al*. 2004). Figure 2.2 shows the comparison between frequencies of MP reported for the three study species and those previously reported in closely related species.

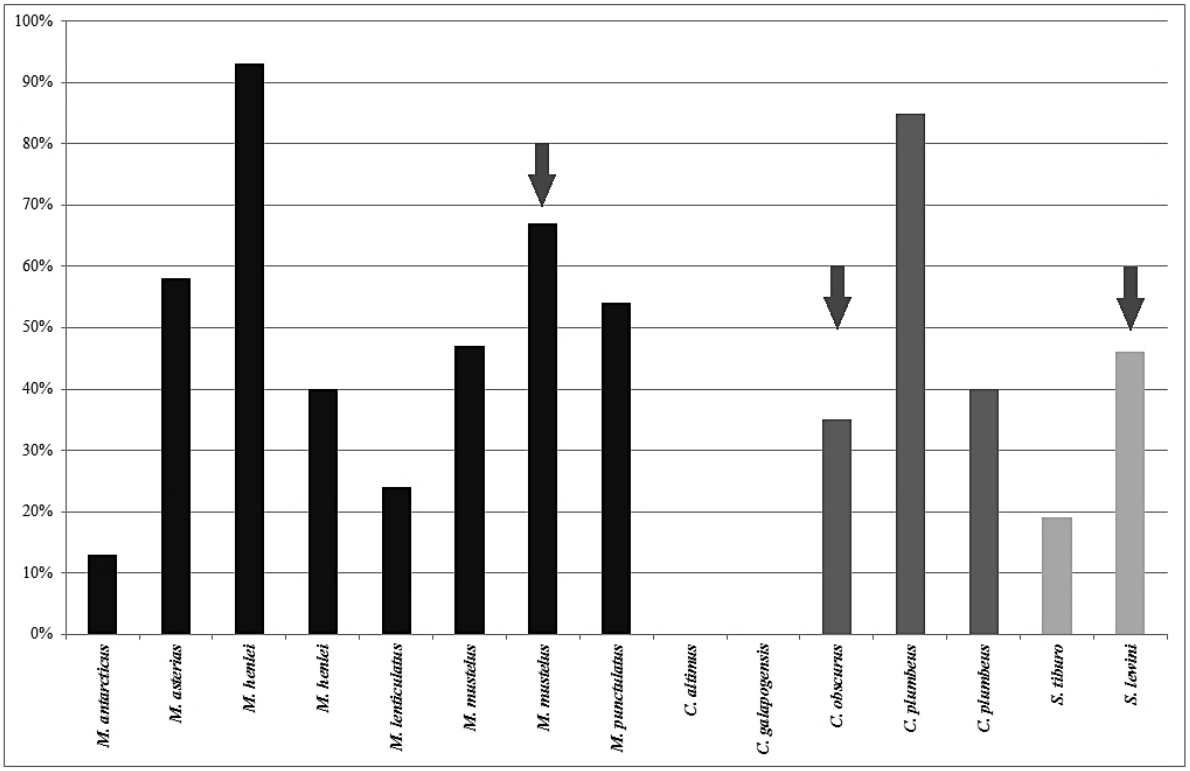

Figure 2.2: Comparison of study species with closely related species.

As is to be expected, the probability of detecting MP within species increased with an increased number of pups per litter as the statistical power of the PRDM software (which makes use of a Monte Carlo algorhythm) increases with an increased litter size. The highest PRDM values were calculated using paternal skews generated in COLONY. When comparing the more conservative parental skews (0,5: 0,5; 0,3: 0,7 and 0,1: 0,9), the highest PRDM was calculated for litters with an evenly distributed paternal skew (0,5: 0,5). In comparison to the results obtained with GERUD software, the rate of MP was higher within each species when calculated using COLONY. This is to be expected as GERUD software uses an exhaustive search algorithm in order to determine the minimum number of sires, whereas COLONY estimates the most likely number of sires.

Despite the relatively high percentage of MP detected in all three species investigated in this study, it is still uncertain what the direct and indirect benefits of MP might be. Studies have

suggested that females might engage in polyandry in order to decrease the chance of inbreeding whilst increasing fecundity and the chance of mating with a male with genes compatible to that of their own (Daly-Engel *et al*. 2006, 2010; Boomer *et al*. 2013). It has also been suggested that MP could contribute to maintaining genetic diversity within a population. There are two main hypotheses regarding the role MP could play in maintaining genetic diversity. One hypothesis suggests that MP increases the effective population size (due to the increase in males that can mate successfully) and thereby reduces the loss of allelic diversity. The more the male engages in polyandrous behaviour, the more offspring he will sire and the greater his reproductive fitness will be (Daly-Engel *et al*. 2006, 2010; Schmidt *et al*. 2010). Furthermore, increased genetic variation within the offspring would increase the likelihood of more offspring being able to survive in fluctuating environmental conditions. Multiple paternity could therefore buffer against the loss of genetic diversity brought on by the slow molecular evolution rate and k-selected life history traits in sharks (Schmidt *et al*. 2010).

Contradictory to this, the second hypothesis states that although MP might increase genetic variability within single litters, effective population size is in fact reduced within populations due to an increase of variable male reproductive success (Daly-Engel *et al*. 2010; Schmidt *et al*. 2010). This is due to males siring less offspring during multiple matings than would have been possible with monandry (Daly-Engel *et al*. 2010; Schmidt *et al*. 2010). In some instances, such as in the case of a population bottleneck, MP can however be highly beneficial in increasing the effective population size of the post-bottleneck populations. It is therefore important to consider the effects that MP has on genetic variation in conjunction with physiological, demographic and behavioural data (Neff & Pitcher 2002).

Studies have shown that male sharks do not engage in parental care or the bringing of nuptial gifts, which could have been a direct benefit to the female (Daly-Engel *et al*. 2010; Schmidt

et al. 2010; Boomer *et al*. 2013). It has been suggested that MP is a by-product of mate encounter rate and avoidance of sexual conflict (Daly-Engel *et al*. 2006; 2010). Globally, *M. mustelus* tend to aggregate in large social groups (Compagno 1984; EFSA 2015). In South Africa, da Silva *et al*. (2013) reported that the species displays site fidelity whilst only travelling short distances between sites with changing seasons. This behaviour along with aggregation in large groups could contribute to the higher percentage of MP seen within *M. mustelus* occurring in South Africa, when compared with the other two study species.

O'Grower (1995) described *C. obscurus* as pelagic, migratory over long distances and likely to segregate according to either size, gender or both. In South Africa, *C. obscurus* caught in the KZN bather protection program show that females significantly outnumber males, which probably reflects the inshore movement of near-term pregnant females to drop their young (Dudley *et al*. 2005) as the core nursery areas for *C. obscurus* are located along the coast of KZN (Hussey *et al*. 2009). Throughout the year males and females migrate along the coast according to prey availability and changes in water temperature (Hussey *et al*. 2009) which makes mate encounters few and widespread (Pratt 1993). Large males are found to peak in the southern region of KZN between June and July, whereas large females are present in the south region between May to July. From March to August large females are found in the northern region of KZN where they most likely migrate to pup (Dudley *et al*. 2005). Migration along the coast throughout the year could lead to fewer and single mate encounters which the female could either accept or dismiss due to their size advantage (Neff & Pitcher 2002; Daly-Engel *et al*. 2010).

Globally, *S. lewini* is considered migratory, but not across large open ocean barriers (Duncan *et al*. 2006) and are dependent on nursery grounds (Duncan *et al*. 2006; Diemer & Hussey 2011). Females have been found to show a strong site fidelity to nursery areas (Daly-Engel *et al*. 2012), whereas males aggregate in order to mate with the females (Baum *et al*. 2007).

Locally, *S. lewini* are widely distributed along the coast of KZN and Transkei, and it is thought that the northern regions of KZN act as nursery ground for this species (Fenessy 1994; de Bruyn *et al*. 2005). Around the coast of KwaZulu-Natal females have been found to frequent waters further offshore than male sharks. It has been theorised that because the females grow larger than males at a younger age they migrate to offshore waters at an earlier age in search of food (de Bruyn *et al*. 2005). This behaviour has also been noted in the north-eastern Brazilian *S. lewini* females who tend to have a preference for deeper offshore areas (Hazin *et al*. 2001). Locally, males have been observed to outnumber females when both genders migrate to inshore regions during the summer months for reproductive purposes. It has been suggested that mating occurs shortly after the females have given birth as the females' ovarian follicles were at maximum diameters when caught during the months of October and March (de Bruyn *et al*. 2005).

Avoiding multiple sexual encounters can be physically tiring for a female (Neff & Pitcher 2002; Saville *et al*. 2002; Schmidt *et al*. 2010; Boomer *et al*. 2013). During copulation the male will bite down on the female's flank or fins in order to stabilise himself enough to wrap his body around the female and successfully insert one of his claspers for insemination. It is common for females to sustain injuries such as open wounds, lacerations and hematomas within the vaginal canal during this process which increases her vulnerability to predation, infection and blood loss (Neff & Pitcher 2002; Daly-Engel *et al*. 2006, 2010). During mating aggregation, males can severely outnumber the female and engage in cooperative behaviour such as herding or mobbing to subdue the female. During these aggregations, uncooperative females can sustain severe injuries and sexually transmitted diseases thereby decreasing the female's fitness with each mating event (Neff & Pitcher 2002; Saville *et al*. 2002; Daly-Engel *et al*. 2010). It is therefore suggested that females engage in polyandry to avoid the physical

costs associated with avoidance rather than benefiting from it directly (Neff & Pitcher 2002; Daly-Engel *et al*. 2006, 2010; Boomer *et al*. 2013).

This hypothesis might also explain the occurrence of variation in MP frequencies between different species as well as between different populations of the same species (Daly-Engel *et al*. 2010; Schmidt *et al*. 2010; Boomer *et al*. 2013). The rate at which females encounter males will differ between species and populations depending on the density of the population as well as the sex ratios. A denser population will have a higher rate of encounter and therefore a higher probability of multiple paternities (Daly-Engel *et al*. 2006, 2010). Sex ratios are important in determining whether a female will copulate as it becomes increasingly difficult and dangerous for the female to refuse the advances of a group of male sharks (Neff & Pitcher 2002; Daly-Engel *et al*. 2010). It has also been suggested that females mate promiscuously because there is little opportunity to evaluate a male's fitness prior to procreation. Mating with several males will therefore increase the likelihood of at least one of the males contributing to an increased offspring survival rate (Daly-Engel *et al*. 2010; Schmidt *et al*. 2010).

Several species of shark also poses the ability to store viable sperm within their shell glands for up to a year. This could allow the sperm to mix and increase the potential of multiple sires (Saville *et al*. 2002; Chapman *et al*. 2004; Daly-Engel *et al*. 2006; Byrne & Avise 2012). It has been suggested that females store the sperm of multiple males in order to induce sperm competition by ways of creating a competitive environment. This sperm competition in turn, will lead to increased fertilisation success or the introduction of more compatible genes. Storing sperm could also decrease the need for mating repeatedly to fertilise all ova during the breeding season, thus having all the genetic benefits of MP whilst decreasing the risk of injury during multiple matings (Neff & Pitcher 2002; Saville *et al*. 2002; Daly-Engel *et al*.

2006). Both *C. obscurus* and *S. lewini* have been shown to store sperm within their nidamental or oviducal glands for extended periods of time (Pratt 1993). Having the ability to store and fertilise eggs without copulation could also indicate that high levels of MP within these species does not necessarily indicate high levels of female polyandry (Boomer *et al*. 2013; Chabot & Haggin 2014). This could be an advantage for these larger predators as distribution of the species is wide and mating opportunities might not be as plentiful as species that aggregate in large groups. Several species of *Mustelus* such as *M. asterias*, *M. antarcticus* and *M. canis* (Hamlett *et al*. 2002; Storrie *et al*. 2008; Farrell *et al*. 2010) have shown the ability to store sperm. Although no sperm storage has been reported in *M. mustelus*, it is possible that *M. mustelus* might also possess the ability to store sperm. Often a paternal skew to one male will be visible in a polyandrous litter when female cryptic choice, male timing or sperm competition has taken place (Boomer *et al*. 2013). Within the *C. obscurus* litters, 50% of litters showed a paternal skew to one male. Within *M. mustelus* and *S. lewini*, the presence of a paternal skew in all the litters was 33% and 36% respectively. It would seem that within *C. obscurus* and *S. lewini*, which have a lower encounter rate than that of *M. mustelus*, there is a higher prevalence of sperm competition or female selection. Hormones stimulate the release of bundles of sperm during ovulation. However, not all sperm stored in the oviducts is released during just one ovulation cycle, indicating that the females do have some control over sperm utilisation (Farrell *et al*. 2014).

This study provides the first evidence for the presence of MP in the three commercially exploited shark species within South Africa, *M. mustelus*, *C. obscurus* and *S. lewini*. The frequency of MP observed between species in this study (*M. mustelus* 67%, *C. obscurus* 35% and *S. lewini* 46%) is consistent with that reported in previous MP studies of sharks. At present it is thought that the occurrence of MP is influenced by a variety of factors such as the occurrence of polyandrous behaviour, reproductive mode differences between sharks species,

the occurrence of sperm storage in some shark species as well as the rate of mate encounter (which in turn is influenced by factors such as population density, aggregative behaviour and site fidelity). *Mustelus mustelus* aggregate in large schools with minimal migration and strong evidence of site fidelity. Due to these large aggregative groups there could be ample time for these sharks to engage in polyandrous behaviour which could lead to the higher MP in *M. mustelus*. It might also be possible for *M. mustelus* to store sperm over long periods of time as many other Mustelus species have this ability. Globally, *C. obscurus* have been documented to segregate according to size, gender or both. Locally, male and female *C. obscurus* tend to frequent the same area for only a short period of time throughout the year during the sardine migration which could reduce the amount of mates encountered throughout the year. Around the coast of South Africa and other parts of the world it has been documented that female *S. lewini* prefer deeper offshore waters, whereas males tend to frequent inshore regions. Females do however show site fidelity and return to the nursing areas to give birth. As male *S. lewini* outnumber females upon their return it could lead to aggregative behaviour which could increase the chances of polyandrous behaviour.

2.5 CONCLUSION

In conclusion, this study demonstrated the usefulness of cross-amplified microsatellite markers in MP assessment in three commercially important shark species and provides further evidence for the high occurrence of MP in sharks as well as the variation found between species. In future, the same microsatellite markers can be used for MP assessment of more populations of the same species or other closely related species of the Carcharhiniformes order. This in turn could provide important baseline data regarding mating behaviour within and between species.

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Chapter 3

Identification of novel molecular genetic markers for two commercially important shark species *Mustelus mustelus* and *Carcharhinus obscurus* using Next-Generation Sequencing platforms

ABSTRACT

An increase in the demand for shark related products coupled with their inherent vulnerability has led to the continuous depletion of shark population's world wide. Determining the extent of population decline and risk of extinction is limited by a lack of species-specific biological data. As the genetic diversity of a population determines how adaptable a population is to a fluctuating environment, it is important to assess the diversity of a population when constructing management strategies. This study therefore aimed to develop species-specific molecular markers for two commercially important shark species in South Africa: *Mustelus mustelus* and *Carcharhinus obscurus*. Molecular markers were identified through reduced genome sequencing of *M. mustelus* on the HiSeq Illumina platform and *C. obscurus* on the Ion Proton platform. For *M. mustelus*, a total of 51,5 million reads were produced and constructed into 27, 5 million contigs. For *C. obscurus*, a total of 27,6 million reads were produced which was constructed into two data sets of 8,9 million and 18,7 million contigs, respectively. A total of 2 700 microsatellite regions and 767 single nucleotide polymorphism (SNP) regions were identified for *M. mustelus*, whereas the *C. obscurus* data set yielded 1 255 microsatellite regions. The amount of microsatellite regions identified is comparable to that of other studies utilizing Illumina based platforms for microsatellite detection.

3.1 INTRODUCTION

3.1.1 Challenges in fisheries management

Due to the exploitation of available stocks, management of fisheries has become exceedingly difficult. The demand for this resource has placed great pressure on the variation and genetic resources of these fishes. Overfishing has led not only to the alteration of the genetic diversity of several fish populations but also driven many thousands of populations to near extinction (Ferguson 1995). Historically, the main focus of fisheries management has been on the economical short-term goal of harvesting from populations with the largest numbers and sized specimens but in the long run this could also lead to the populations being depleted (Ward & Grewe 1995). Shark populations have been fished on a commercial scale since the 1920s, with global catches totalling 270 000 tons by the 1950s and tripling by the year 2000. The drastic decline of these shark populations can be attributed to three main factors: (i) Sharks follow a K-selected life strategy (they produce less eggs, are less fertile, have relatively slow growth rates and reach sexual maturity at a later age in comparison to teleost fishes) (Musick *et al*. 2000; Stevens *et al*. 2000); (ii) species identification guides and protocols are poorly set up (data on shark landings are not species-specific but rather grouped together in morphologically similar species or families) and (iii) in comparison to teleost fishes, sharks have a low economic value which requires that more sharks have to be fished in order to obtain the same profit (da Silva *et al*. 2015). The above mentioned factors can contribute to overfishing in the following ways: firstly, having a K-selected life strategy makes sharks vulnerable to overfishing as these populations cannot replenish their numbers as quickly as teleost fishes; secondly, each species is uniquely adapted and will have varying capabilities of coping with the loss of large numbers of animals (one species might be able to cope with the loss in numbers due to adaptations while another species with different adaptations might not); and thirdly fishing more sharks to achieve the same economic value will lead to a great reduction in population sizes which could lead to a loss of genetic diversity within these populations. It is therefore vital for fisheries to incorporate molecular genetics research in the construction of long-term sustainable fisheries management programs (Park & Moran 1995).

3.1.2 The role of genetic variation

Genetic variation within populations enables the population to adapt to fluctuating environments. Without it, a population will be unable to adapt which will ultimately lead to the extinction of the population. Novel genetic variation is introduced into a population by means of mutation (which can create new alleles) or through the introduction of new alleles into the gene pool by immigration of new individuals into the population. Genetic variation within a population can therefore be measured in the abundance of various alleles within a populations' gene pool (Çiftci & Okumus 2002). By removing large amounts of individuals from a population through commercial fisheries the amount of individuals who could contribute unique alleles to the populations' gene pool are lost. This in turn will reduce the populations' ability to adapt in an ever changing environment. Low productivity such as late sexual maturity, small litters, long inter-birth intervals and slow growth rates affects how well a species is able to adapt to increased mortality rates. As sharks are prone to low productivity, they can be seen as extremely vulnerable to extinction (Garcia *et al*. 2008).

As each population has a unique gene pool it is important to manage each population according to the amount of genetic diversity it holds. Distinguishing between different populations or stocks has however proven difficult for fisheries management (Çiftci & Okumus 2002). For the most part, fish stocks have been defined as a group of fish harvested by a specific method or in a specific location (Carvalho & Hauser 1995). It would be more beneficial to group these stocks in terms of genetic distinctiveness rather than just grouping stocks according to a specific harvesting location. In order to understand the genetic diversity within a stock, several influences such as ecological processes, mating strategies, selection, genetic drift as well as migration will need to be taken into account (Çiftci & Okumus 2002). Once the genetic diversity and uniqueness of a population has been established, management decisions such as not harvesting from an already genetically weak or inbred population can be made (Çiftci & Okumus 2002). Maintaining high levels of genetic diversity within populations is crucial for preserving these stock resources. In order to evaluate genetic diversity within populations, neutral polymorphic molecular markers need to be utilized (O'Connell & Wright 1997; Schlötterer 2004). Genetic markers can be defined as heritable polymorphisms within an organism's DNA which can be detected in one or more populations (Vignal *et al*. 2002; Davey *et al*. 2011). On a molecular level, only three main types of markers exist: variations in number of tandem repeats (VNTRs), single nucleotide changes (SNPs) and insertions or deletions (Indels) (Vignal *et al*. 2002). At first, genetic diversity was

studied through the use of blood group polymorphisms and protein gel electrophoresis (O'Connell & Wright 1997; Schlötterer 2004) but this was soon followed by a boom in the discovery of more efficient molecular markers (O'Connell & Wright 1997; Vignal *et al*. 2002; Schlötterer 2004) with microsatellite markers being the most abundantly used markers in fisheries management (Vignal *et al*. 2002; Barbara *et al*. 2007). Microsatellite and SNP markers are frequently used to answer questions concerning population ecology, conservation genetics and evolution as they are not only the most abundant and wide spread markers throughout the genome, but also the most polymorphic and therefore the most informative markers (Lui & Cordes 2004; Morin *et al*. 2004).

3.1.3 Molecular markers

Microsatellite markers comprise of multiple copies of tandem organized simple sequence repeats (SSRs) or short tandem repeat units (Wei *et al*. 2014) that range in size from one to six base pairs (Liu & Cordes 2004). These markers can differ in the types of repeats such as GA repeats interposed with GT repeats (Wright & Bentzen 1994). Within genomes, several imperfect microsatellite markers can also be found. These microsatellite regions contain indels and substitutions of nucleotides (for example CGCGCGACGCGCG), making them imperfect (Mudunuri & Nagarajaram 2007). Microsatellite sequences are abundant, highly polymorphic and distributed throughout eukaryote and prokaryote genomes (Zane *et al*. 2002; Schlötterer 2004; Wei *et al*. 2014). In fish species, microsatellites have been reported to occur as often as once every 10 kilobases which make them relatively easy to isolate (Wright & Bentzen 1994; Lui & Cordes 2004). These markers usually have a high degree of length polymorphism and are found in both non-coding as well as coding regions (Zane *et al*. 2002). Microsatellites are present on all chromosomes and tend to be evenly distributed in most regions of the chromosomes (Lui & Cordes 2004). Microsatellite polymorphisms are caused by slippage or slipped-strand mismatching events during the replication of DNA as they are highly susceptible to length mutations (Wright & Bentzen 1994; Zane *et al*. 2002; Schlötterer 2004). Due to these frequent mutations microsatellite alleles are highly variable and can contribute to high levels of observed heterozygosity within species (Wright & Bentzen 1994). Usually, highly polymorphic microsatellite markers are the markers with larger numbers of repeated units, although microsatellites with as little as five repeats have been shown to be polymorphic (Lui & Cordes 2004). The relatively small size of these marker loci makes them suitable for genotyping using PCR methods (Lui & Cordes 2004). Although microsatellite flanking primers are straightforward and easy to develop, it can be time-consuming and

costly. Alternatively, microsatellites can be applied between closely related species although the success rate decreases with genetic distance between these species (Arif *et al*. 2010). Nuclear microsatellite markers are useful in species where mtDNA variation is low due to their greater variability in comparison to mtDNA and allozymes (Heist & Gold 1991).

Commonly, microsatellite markers were developed using genomic libraries enriched for repetitive motifs. These libraries were then cloned and hybridized to search for positive clones which were isolated by plasmids and sequenced. Finally, primers were designed from flanking regions and the microsatellites evaluated for polymorphism (Hoffman & Nichols 2011; Silva *et al*. 2013). Although this is a simple process, success rate depends on the efficiency of the enrichment and cloning protocols. These protocols can be greatly inefficient with positive clone yields averaging as little as 2 - 3%. Furthermore, marker isolation from species with a small variety of microsatellites is highly problematic (Hoffman & Nichols 2011). Identification and sequencing of the flanking regions for each potential microsatellite marker has to be performed which could also be costly. More recent methods included microsatellite-enriched genomic DNA libraries which were made in order to develop a higher number of potential markers (Lui & Cordes 2004; Silva *et al*. 2013) but even potential microsatellites (loci) that did amplify successfully could turn out to be monomorphic. This is regularly observed in species that inherently have low levels of genetic diversity (Hoffman & Nichols 2011).

The major disadvantage of microsatellites when it comes to newly examined species is the need to be isolated *de novo* (Hoffman & Nichols 2011). Nucleotide substitution rates are higher in non-coding regions than in coding regions. Microsatellites are more problematic than mtDNA when it comes to the strategy of designing universal primers to match the conserved sequences. However, in cetaceans, turtles and fish, higher conserved flanking regions for some microsatellite loci have been reported (Zane *et al*. 2002). This allows for cross-species amplification. When orthologous loci are tested in other species, this bias could lead to a lower level of polymorphism observed than in the focal species. Observing high polymorphism in species therefore does not guarantee finding a similar level of polymorphism in related species, particularly when evolutionary distances between species increase. Fortunately for closely related taxa, like species belonging to the same genus or currently separated genera, a relatively high success rate with cross-species amplification has been reported (Zane *et al*. 2002).

With the cost reduction in Next-Generation Sequencing technology (NGS) it has become possible to efficiently and cost effectively devellop microsatellite markers without prior knowledge of a species genetic make-up (Wei *et al*. 2014; Fernandez-Silva *et al*. 2013). NGS platforms such as 454 Roche, MiSeq and HiSeq Illumina as well as Ion Torrent PGM and Proton platforms have been successfully utilized to identify hundreds to thousands of possible microsatellite loci per species (Wei *et al*. 2014). This is possible due to the larger fragment sizes that can be evaluated for possible microsatellite regions. These larger regions enable primers to be easily designed as sufficient flanking regions on both sides of the microsatellite region is present. When comparing NGS sequencing to enrichment methods, NGS platforms have several advantages to identifying microsatellite loci. Although enrichment protocols only target a few motifs, these motifs can differ largely amongst different taxa. With NGS, no prior motif selection is necessary. As the restriction enzymes used to fragment the DNA can cut into transposable elements, these repetitive elements could be over represented within the fragments. If the microsatellites selected do fall within these repetitive regions it could render the recovered loci unusable. Finally microsatellite development using NGS platforms can be more cost effective, faster and yield more loci than conventional enrichment protocols (Gardner *et al*. 2011).

Single nucleotide polymorphisms (SNPs) are single changes in nucleotide composition brought on by point mutations which cause a change in bases at a nucleotide position within a locus giving rise to different alleles and can be found every 0.3-1kb (Lui & Cordes 2004; Fromentin *et al*. 2009). SNPs are co-dominantly inherited, bi-allelic markers (usually two purines, A/G, or two pyrimidines, C/T) but can have as many as four alleles at a given locus. Although microsatellite markers have higher polymorphic information content (PIC) due to their multi-allelic nature, SNPs, with their bi-allelic nature, can be equally as informative when used in larger numbers (Lui & Cordes 2004). Since the start of DNA sequencing in 1977 it has been possible to identify these base substitutions but the development of gene chip technology in the 1990s allowed for the actual genotyping of these polymorphic markers (Lui & Cordes 2004). The use of SNP markers in studies has grown over the past few years as these markers are suitable for automation, highly abundant in most organisms, can be used in genome-wide scans, can possess high information content and can serve as powerful analytical tools for various genetic applications such as identification of candidate genes for QTL and genome mapping (Lui & Cordes 2004; Morin *et al*. 2009). In comparison to microsatellite loci, SNPs have a lower rate of mutation and variability (Schlötterer 2004;

Fromentin *et al*. 2009; Morin *et al*. 2009), which eliminates the occurrence of homoplasy (Seddon *et al*. 2005), possess simple mutation models of evolution, which are better understood (Ryynanen *et al*. 2007), and are widespread and abundant in many species' genomes (Fromentin *et al*. 2009; Morin *et al*. 2009).

Since DNA sequencing was the most popular approach for isolating SNPs, several methods such as expressed sequence tag (EST) analysis, random shotgun sequencing as well as PCR amplicon sequencing were developed for this purpose. For large scale SNP discovery, more robust methods such as pyro-sequencing (Ahmadian *et al*. 2000; Alderborn *et al*. 2000), technology were used. For larger and more complex genomes, pyro-sequencing proved to be too expensive and time consuming. It was necessary to reduce the sheer size of the genome before sequencing could take place. Restriction enzymes can be used to fragment DNA into smaller segments which can be pooled and sequenced in parallel to capture a targeted segment of the genome whilst still maintaining sufficient coverage of the sequence (Mascher *et al*. 2013). Due to the decreased cost of NGS platforms it is now possible to produce millions of sequence reads which can be utilized to identify vast amounts of molecular genetic markers (Everett *et al*. 2011; Milano *et al*. 2011). Although expressed sequence tags (ESTs) have mostly been used to identify microsatellite markers, the use of these sequences to identify SNPs with has grown in popularity. This is because these genetic variants are more widespread and abundant in the genome in comparison to microsatellite regions (Milano *et al*. 2011). For non-model organisms (organisms with no reference genome), genome segments of multiple individuals have been screened in order to discover SNPs. As many species share conserved sequences between them, it is possible to compare these sequences from different individuals in order to detect base changes between them (Morin *et al*. 2004).

In order to identify SNP regions within an organism, contigs need to be constructed and mapped against a reference genome or scaffold which requires sufficient genome coverage (Everett *et al*. 2011; Nielsen *et al*. 2011). Variations in nucleotides of mapped sequences can then be identified as possible SNP regions. In order to identify true SNPs within mapped contigs the contigs need to be aligned as accurately as possible. Most NGS alignment algorithms are based on the "Burrows-Wheeler transform" (compression of data) such as BWA, SOAP and BOWTIE, which are less stringent than hashing algorithms (data string is converted into a numeric string) such as STAMPY and MAQ12 (Nielsen *et al*. 2011). The SNP density across a species genome will determine the number of genome segments that

need to be screened in order to discover SNPs. For example, if SNPs occur every 200-500bp in order to yield 50 SNPs from a genome, 100 genome segments of 800bp long should be screened (Morin *et al*. 2004). It is generally more difficult to align sequenced regions that differ considerably from the reference genome but this can be mitigated through the use of paired-end or longer reads (Nielsen *et al*. 2011). With the growing effectiveness of NGS technology and improved bioinformatics tools to analyse the raw sequence reads produced, wide-spread SNP identification is becoming increasingly plausible (Milano *et al*. 2011).

In comparison to microsatellites, a relatively large number of SNPs need to be isolated due to their bi-allelic nature (Morin *et al*. 2004). For this, NGS technology has recently been used to generate sequencing data from which SNPs and microsatellite regions could be identified on a genome wide basis. By implementing NGS technology, thousands of potential molecular marker regions can be identified for primer design and amplification (Silva *et al*. 2013). Although it is no longer difficult to identify potential molecular marker regions using NGS technology, it still remains costly and laborious to validate these identified regions. Many platforms such as Ion Proton and Torrent PGM, MiSeq and HiSeq Illumina and Roche 454 have been implemented for molecular marker identification, with Illumina and Roche 454 being the most popularly used platforms (Wei *et al*. 2014). The Roche 454 platform incorporates a pyro-sequencing method which entails the measuring of pyrophosphate release as nucleotides are incorporated into the template strands. Nucleotides are sequentially injected in the reaction mixture and successful incorporations are recorded (Vignal *et al*. 2002; Voelkerding *et al*. 2009). Both the Ion Proton/Torrent PGM and Illumina platforms follow a sequence-by-synthesis approach. For Illumina base-by-base sequencing takes place as fluorescently labelled nucleotides are washed over the wells containing the fragmented DNA. As each nucleotide is incorporated into the template, a fluorescent signal is produced which is recorded (Illumina Incorporation 2013). For the Ion Proton/Torrent PGM nucleotides are incorporated sequentially with a change in pH being recorded as the hydrogen ion is released during incorporation. The release of a hydrogen ion changes the chemical composition of the well (Fu *et al*. 2013; Glenn 2011). In comparison to earlier isolation methods, NGS platforms can identify hundreds to thousands of potential molecular markers per species (Wei *et al*. 2014).

3.1.4 Research rationale

Although chondrichthyan species have been well researched within South Africa, these studies have mostly focused on telemetry, distribution and abundance of non-harvested species such as the ragged-tooth shark *Carcharias Taurus* and the great white shark *Carcharodon carcharias*. Very few studies have been done on the impact of fisheries on the commercially important sharks within South Africa (da Silva *et al*. 2015). To date there are no microsatellite or SNP markers available for most of the commercially exploited shark species within South Africa. At present, 15 microsatellite markers have been isolated for scalloped hammerhead sharks *Sphyrna lewini* (Nance *et al*. 2009). No microsatellite markers, however, exist for *M. mustelus* or *C. obscurus*. This study therefore aimed to identify novel microsatellite and SNP markers for both the *M. mustelus* and *C. obscurus* species through the use of NGS and appropriate downstream analysis pipelines. Once identified, these markers could contribute to the growing amount of genetic markers available which can be utilized for studies investigating population diversity and reproductive mode.

3.2 MATERIALS AND METHODS

3.2.1 Sequencing of species

One *Mustelus mustelus* and one *Carcharhinus obscurus* sample with a DNA concentration of 500ng/µL were sent for Next-Generation Sequencing using a HiSeq Illumina and Ion Proton based platform respectively. For *M. mustelus* 2 X 250bp paired-end sequencing was performed. For *C. obscurus*, the same sample was digested for 90 minutes and 180 minutes, respectively with both *EcoR1* and *Mse1* enzymes to create blunt-ended fragments. Ion-compatible barcode adapters were then ligated onto the digested DNA fragments. Fragment sizes between 250-300bp were selected for sequencing on the Ion Proton platform.

3.2.2 Processing of raw Next-Generation Sequencing data

The sequencing data for *M. mustelus* and *C. obscurus* was obtained in fastq file format (text-based files which store both nucleotide sequences and their quality scores) from the ARC (University of Pretoria) and CAF (University of Stellenbosch) respectively. Files were uploaded to the Stellenbosch University High-Performance Computing (HPC) cluster for processing. Data files were in fastq.gz format and had to be unzipped to fastq format using

the unpigz command. All fastq files were run through FASTQC 3.0 software (Patel & Jain 2012) as well as PRINSEQ (0.20.4) software (Schmieder & Edwards 2011) using default parameters in order to evaluate the quality of the data. For the FASTQC software no parameters needed to be specified, only the location of the input file. For PRINSEQ default parameters included –graph_data, -i –html_all which first evaluated the quality of the data and reported on it in graph format (-graph_data) and then converted the graph files (-i) into a http link (-html_all) in order to view them. For *M. mustelus* data, FASTQC and PRINSEQ analysis reported the presence of poly A-tails, Nextera adapters, overrepresented sequences, and some lower quality reads as well as unidentified nucleotides (N) within the reads. For *C. obscurus*, Ion Proton software was used by the service provider for adaptor, poly-A tail trimming and quality filtering.

Mustelus mustelus reads were run through the software TRIMMOMATIC (0.33) (Bolger *et al*. 2014). TRIMMOMATIC was used to remove all Nextera adapters with 2:30:10 parameters (TRIMMOMATIC searches for seed matches with a maximum mismatch of 2, extends and clips seeds when a score of 30 is reached for paired-end reads and 10 for single ended reads), perform a sliding window function with parameters 4:15, removed 4bp from both the leading as well as trailing reads if the read was below the quality threshold (phred score 15) as well as discarding reads below 36 base pairs in length. TRIMMOMATIC yielded 5, 23 GB of fastq files for both forward as well as reverse reads. Forward and reverse reads were then combined into a single fastq file using the cat command. FASTQC 3.0 software (Patel & Jain 2012) was run to evaluate reads before proceeding. FASTQC results revealed both the absence of adapters as well as poor quality reads. The reads still contained overrepresented sequences which were then blasted against the NCBI database.

Since the majority of the overrepresented reads were from the sunflower species *Helianthus maximiliani*, the fastq file was mapped against the *H. maximiliani* genome using BOWTIE 2.2.5 software (Langmead 2010) using the command aln which maps the input file against the reference genome. The *H. maximiliani* reads were then extracted from the fastq file using a FASOMERECORDS script (Kent 2011) called fastq-filter_extract_reads.pl which extracted all reads with *H. maximiliani* identifiers (IDs) and placed them in a separate file from the *M. mustelus* reads. The fastq file containing the *M. mustelus* reads (16 GB) was then further processed to identify potential microsatellite markers and single nucleotide polymorphism (SNP) loci.

3.2.3 Identification of possible microsatellite markers

The *M. mustelus* fastq file was then run through ABYSS 1.5.2 software (Simpson *et al*. 2009) in order to construct contigs from the reads. *Carcharhinus obscurus* reads were assembled into contigs using the software MIRA 4.0.5. (Chevreux *et al*. 1999). One *M. mustelus* (1. 5 GB) and two *C. obscurus* contig files (1, 7 GB and 3, 5 GB) of data were successfully constructed. The two *C. obscurus* files were kept separate in order to compare the influence of digestion time on sequencing output. The software MISA 1.0 (Thiel *et al*. 2003) was used after modifying the MISA.pl script to read the MISA.ini file from the command line as an argument in order to identify possible microsatellite positions. The *M. mustelus* contig file was run through MISA software in order to search for perfect repeats. Contigs larger than 300bp for *M. mustelus* and contigs larger than 250bp for *C. obscurus* were selected for analysis in MISA (Thiel *et al*. 2003). Contigs larger than 250-300bp were extracted from the fasta files using the software BIOAWK (Aho *et al*. 1988) and placed in separate files for analysis. Once these candidate microsatellite marker files were constructed, contigs with potential microsatellite loci towards the middle of the contig (larger than two nucleotide repeats) were manually selected in MICROSOFT EXCEL (Microsoft 2010). The selected contigs' IDs were then used to construct a file using a PERL 5.20.2 (Wall & Loukides 2000) and FASOMERECORDS script (Kent 2011). This contig file was then blasted against the NCBI database to filter out the contigs which contained hits with microsatellites against other elasmobranch or pisces species. The software PRIMER3 (v. 0.4.0) (Untergrasser *et al*. 2012) was then used to design primers for the selected candidate microsatellite loci.

3.2.4 Validation of microsatellite markers

Primer pairs were designed with open-source software PRIMER3 (Untergasser *et al*. 2012) for fifteen *M. mustelus* and fourteen *C. obscurus* candidate microsatellite loci respectively. Polymerase chain reactions (PCR) were performed to test for amplification success of all primer pairs. A 10 µL reaction mixture was prepared containing 1 µL (50 ng/µL) of genomic DNA, 1x Buffer, 200 µM dNTPs, 0,2 µM of forward and reverse primers, 1,5 mM $MgCl_2$ and 0,1 units of Taq polymerase. The 29 candidate markers were optimized by conducting a gradient PCR in order to establish annealing temperatures for each primer pair. PCR cycling conditions included an initial activation step of one cycle at 95°C for 2 minutes followed by 35 cycles of a denaturing step at 94°C for 30 seconds, annealing step at 53-59°C for 60 seconds and extension step at 72°C for 2 minutes. A final extension step at 60°C for 5

minutes was conducted. After annealing temperatures had been established for all markers, DNA of four individuals was amplified in a preliminary attempt to test for polymorphism within species. PCRs were performed in a GeneAmp 2700 thermal cycler and PCR products were visualised at on a 3% agarose gel run at 70 volts.

3.2.5 Identification of single nucleotide polymorphism markers

At present there are very few shark species with a complete draft genome available. Fortunately a reduced Australian ghost shark *Callorhinchus milii* genome is publicly available and was downloaded from the NCBI database. An index file of the *C. milli* genome was constructed using BWA 0.7.1 software (Li & Durbin 2009). The fastq files containing *M. mustelus* and *C. obscurus* reads were then mapped against the *C. milii* genome using BWA software which resulted in .sai files (indexed sam files). The .sai files were converted to .sam files (tab-delimited text file containing sequence alignment data) using the sampe command in BWA. The software SAMTOOLS 1.2 (Li *et al*. 2009) was then used to construct an index file from the *C. milli* reference genome using the faidx command. The indexed reference genome was finally converted into a .bam file (binary version of .sam files) along with the mapped reads .sam file into a single .bam file for each species using the import command. These files were then sorted and indexed before variant calling files were generated for both species using the mpileup command also in SAMTOOLS. The variant calling files were then processed using BCFTOOLS 0.1.12 software (Li *et al*. 2009) which resulted in files containing the identified variants in bcf format (variant call file in binary format). Variant files were then filtered using BCFTOOLS as well as VCFUTILS (Danecek *et al*. 2011) to create a final variant vcf file (text-based variant call format file) from which single nucleotide polymorphisms could be identified for each species.

3.3 RESULTS

3.3.1 Processing of raw Next-Generation Sequencing data

Approximately 35 GB of read data was received from the service provider. A total of 4 million reads, with an average length of 250bp were identified within each of the 26 pair-end files. After trimming and quality filtering, remaining reads were compiled into single fasta

file with a total of 51 599 112 reads. For both data sets (assembled read files for both *M. mustelus* and *C. obscurus*) sequence lengths ranged from 36-250bp with a presence of N nucleotides and adapters at 0.00%.

3.3.2 Microsatellite detection

The search for microsatellite regions was performed on the data sets for *M. mustelus* and *C. obscurus*. The *M. mustelus* dataset contained a total of 27 512 666 contigs, whereas the two *C. obscurus* data sets contained 8 927 950 contigs for the 180 minute digestion sample and 18 757 505 contigs for the 90 minute digestion sample. In order to search for perfect microsatellite regions, contigs larger than 250bp were extracted from the *M. mustelus* and *C. obscurus* data sets. For *C. obscurus*, the two datasets generated from the one sequenced sample were kept separate in order to compare whether the digestion duration had an effect on the amount of microsatellite regions identified. Searches for microsatellite containing regions were restricted to perfect microsatellite motifs. For *M. mustelus*, 2 700 microsatellite regions were identified, whereas 1 255 microsatellite regions were identified for *C. obscurus* digested for 90 minutes and 150 microsatellite regions were identified for *C. obscurus* digested for 180 minutes. Table 3.1 shows the results of the microsatellite region searches performed on both the aforementioned files. The most abundant microsatellite regions were di-nucleotides (1 629 for *M. mustelus* and 147 for *C. obscurus*), followed by tri-nucleotides (232 for *M. mustelus* and 15 for *C. obscurus*).

Identified microsatellite regions from the contigs larger than 250bp were then selected for primer design for each species. Microsatellite regions for primer design were selected based on the position of the microsatellite motif within the contigs. Motifs that were found to be too close to the start or end of the contig were excluded from further processing. For *M. mustelus*, 83 contigs were selected whereas for *C. obscurus* 90 contigs were selected to BLAST against the NCBI database to ensure contig similarity to either shark or teleost sequences. For both species most BLAST search results returned a positive blast against a bacterial species. For *M. mustelus,* 11 contigs returned positive BLAST results for fish species and 11 contigs returning positive BLAST results for shark species. For *C. obscurus* five contigs blasted against teleost species and 18 contigs blasted against shark species. Fifteen microsatellite-containing regions for *M. mustelus* and fourteen for *C. obscurus* were selected for primer design. Table 3.2 shows the regions selected for primer design as well as repeat motifs and blast results.

Table 3.1: Summary of a microsatellite region search performed in MISA for *M. mustelus* and *C. obscurus* contigs larger than 300bp and 250bp respectively.

	M. mustelus contigs larger than 300bp	*C. obscurus* contigs larger than 250bp digested 180 minutes	*C. obscurus* contigs larger than 250bp digested 90 minutes
Total number of sequences examined	82 879	927	35 661
Total size of examined sequences	33 407 493bp	243 692bp	9 445 677bp
Total number of identified SSRs	2 700	150	1 255
Number of SSR containing sequences	2 572	121	1 099
Number of sequences containing more than one SSR	120	26	116
Number of SSRs present in compound formation	47	29	141
Di-nucleotides	1 629	3	147
Tri-nucleotides	232	-	15
Tetra-nucleotides	31	-	6
Penta-nucleotides	-	-	2

Table 3.2: Microsatellite regions selected for *M. mustelus* and *C. obscurus* including repeat motif, size, and position in contig and blast search results.

Species	Contig ID	SSR type	SSR	Size in bp	Start	End	Blast search result
M. mustelus	136194_818_1714	p2	$(TC)_6$	12	561	572	*Protopolystoma xenopodis* *
	603691_889_5405	p2	$(AT)_7$	14	651	664	*Danio rerio*
	17731961_828_2935	p2	$(AC)_6$	12	756	767	*Cyprinus carpio*
	19222092_770_1754	p2	$(TC)_7$	14	208	221	*Astyanax mexicanus*
	23023355_739_1638	p2	$(TC)_6$	12	399	410	*Camelus dromedaries* *
	26193882_735_1896	p2	$(CT)_6$	12	475	486	*Cucumis sativus* *
	26403340_740_2107	p2	$(TG)_7$	14	481	494	*Astyanax mexicanus*
	5551050_1405_8499	p3	$(GTC)_7$	21	885	905	*Ochrobactrum anthropi* *

	12961358_707_1755	p3	$(CTC)_6$	18	452	469	*Callorhinchus milii*
	14682365_1857_7998	p3	$(CGC)_6$	18	1491	1508	*Chrysemys picta bellii*
	18961995_1089_3100	p3	$(GCT)_5$	15	903	917	*Callorhinchus milii*
	26836431_1069_3092	p3	$(CAG)_5$	15	548	562	*Callorhinchus milii*
	7951092	p4	$(GAAT)_5$	19	253	272	*Callorhinchus milii*
	11748443	p4	$(CATA)_5$	19	329	348	*Squalus acanthias*
	61216	p3	$(CAA)_5$	14	327	341	*Callorhinchus milii*
	1178354	p3	$(GAT)_6$	17	241	258	*Callorhinchus milii*
	14447036	p3	$(GCA)_5$	14	103	117	*Squalus acanthias*
	12929751	p3	$(AGC)_6$	17	265	282	*Callorhinchus milii*
	14824632	p3	$(CTC)_5$	14	352	366	*Squalus acanthias*
C. obscurus	45LXA:10554:09194	p2	$(AG)_6$	11	209	198	*Galeocerdo cuvier*
	45LXA:09263:12443	p2	$(GA)_{12}$	23	203	180	*Scyliorhinus canicula*
	45LXA:03070:04813	p5	$(TAGTC)_5$	24	123	99	*Heterodontus francisci*
	45LXA:04796:12627	p5	$(ATTTG)_5$	24	136	112	*Callorhinchus milii*
	45LXA:07096:09089	p4	$(TTCA)_5$	19	124	105	*Scyliorhinus canicula*
	45LXA:05232:05820	p4	$(GGCA)_6$	23	155	132	*Ginglymostoma cirratum*
	45LXA:09892:10714	p3	$(GGA)_5$	14	190	176	*Squalus acanthias*
	45LXA:06987:06961	p3	$(AAG)_5$	14	119	105	*Heligmosomoides polygyrus*
	45LXA:06809:09492	p3	$(TTA)_7$	20	133	113	*Schistosoma rodhaini*
	45LXA:07901:08045	p3	$(ATT)_5$	14	124	110	*Hexanchus griseus*
	45LXA:04481:12308	p3	$(ACA)_5$	14	159	145	*Scyliorhinus canicula*
	45LXA:04849:07121	p3	$(CTC)_5$	14	137	123	*Trichobilharzia regenti*
	45LXA:02939:05771	p2	$(AC)_6$	12	109	97	*Scyliorhinus canicula*
	45LXA:09925:04970	p2	$(TC)_6$	12	116	104	*Squalus acanthias*

In total, primers were designed for 29 candidate microsatellite loci and are shown in Table 3.3 in Appendix A. This Table also includes contig ID, product size, melting temperature, percentage of GC content, maximum intra-pair difference as well as maximum self-complementarity score.

3.3.3 Validation of microsatellite markers

Primers were designed for 15 microsatellite regions for *M. mustelus* and 14 microsatellite regions for *C. obscurus*. Markers were optimized through the use of a gradient PCR in order to determine each primer pair's annealing temperature. Table 3.4 shows each marker's annealing temperature after optimization. From the 15 microsatellites selected, all 15

M. mustelus microsatellite markers amplified successfully, whereas only eight markers (*CO005*, *CO006*, *CO007*, *CO008*, *CO009*, *CO011*, *CO012* and *CO014*) successfully amplified for *C. obscurus*. From the six markers that did not amplify successfully, two markers (*CO001* and *CO002*) amplified non-specifically whereas four markers (*CO005*, *CO006*, *CO010* and *CO013*) showed no amplification.

Table 3.4: Optimized annealing temperatures for each *M. mustelus* and *C. obscurus* primer pairs tested. (-) indicates unsuccessful amplification of primer pair.

	Marker	Annealing temperature °C		Marker	Annealing temperature °C
M. mustelus	*MM001*	57	***C. obscurus***	*CO001*	-
	MM002	56		*CO002*	-
	MM003	56		*CO003*	56
	MM004	56		*CO004*	58
	MM005	57		*CO005*	-
	MM006	57		*CO006*	-
	MM007	57		*CO007*	56
	MM008	56		*CO008*	56
	MM009	56		*CO009*	55
	MM010	56		*CO010*	-
	MM011	56		*CO011*	55
	MM012	56		*CO012*	59
	MM013	56		*CO013*	-
	MM014	58		*CO014*	55
	MM015	58			

Once annealing temperatures were established for each marker a PCR was run using all successfully amplified markers for both species. PCR product was then run on a 3% agarose gel on 70 volts.

3.3.4 Identification of single nucleotide polymorphism markers

Because no reference genome is available for *M. mustelus* or *C. obscurus*, the genome of the Australian ghost shark *Callorhinchus milii* was used in an attempt to assemble the *M. mustelus* and *C. obscurus* reads. The *C. milli* genome contains 13,9 million reads to which the 51 million *M. mustelus* and 18 million *C. obscurus* reads were mapped against using the BWA software. Identification of possible SNP regions with SAMTOOLS resulted in 7 200 potential SNP regions for *M. mustelus* and 4 700 potential SNP regions for *C. obscurus*. The

software BCFTOOLS was used in order to identify possible SNP regions within the mapped reads for both species. After filtering SNPs with phred scores below 100, only *M. mustelus* retained SNP data while *C. obscurus* had no identified SNPs after as all identified SNPs had a phred score lower than 100. The retained SNPs for *M. mustelus* is summarised in Table 3.5. Heterozygous SNPs carried one copy of the reference nucleotide as well as one copy of the alternative nucleotide. The homozygous SNPs carry two copies of the alternative nucleotides and no copy of the reference nucleotide. Table 3.6 gives a summary of the nucleotides that substituted the reference sequence for *M. mustelus* as well as the frequency for both the homozygous as well as heterozygous SNPs. For the *M. mustelus* multi-allelic SNPs, the frequency of nucleotide combinations are represented in Figure 3.1 for these SNPs.

Table 3.5: SNP statistics for *M. mustelus* species.

	Filtered SNPs
Number of SNPs	767
Number of indels	0
Number of multi-allelic sites	19
Number of heterozygous SNPs	256
Number of homozygous SNPs	491
Number of multi-allelic SNP sites	19

Table 3.6: Frequency of *M. mustelus* nucleotide substitutions in identified homozygous and heterozygous SNPs.

Heterozygous SNPs					
Reference nucleotide		A (65)	C (57)	G (66)	T (68)
Substitutions:					
	A	-	14.04%	30.30%	44.12%
	C	24.6%	-	57.57%	29.41%
	G	38.5%	49.12%	-	26.47%
	T	36.9%	36.84%	12.13%	-
Homozygous SNPs					
Reference nucleotide		A (137)	C (103)	G (119)	T (132)
Substitutions:					
	A	-	13.59%	35.29%	28.04%
	C	23.36%	-	42.85%	47.72%
	G	50.36%	55.34%	-	24.24%
	T	26.28%	31.07%	21.85%	-

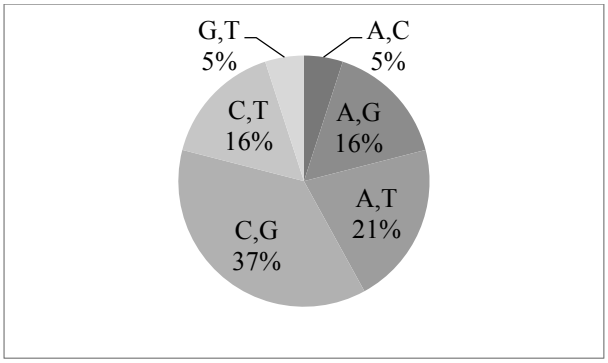

Figure 3.1: Frequency of *M. mustelus* nucleotide combinations for multi-allelic SNPs.

3.4 DISCUSSION

Genetic diversity within populations is determined by the variation of the alleles present in the effective breeding population. These genetically distinct groups of animals can be seen as different stock populations within the same species and should be managed independently according to the unique genetic diversity within specific populations or stocks (Abdul-Muneer 2014). As genetic diversity plays a key role in the survival of a population, it is an important aspect to consider within a management or conservation program for a particular species. The use of molecular genetic markers has made it possible to assess the genetic diversity within species, populations and individuals (Chauhan & Rajiv 2010). The past 50 years has seen an increase in the use of molecular markers in various applications pertaining to the management and conservation of species biodiversity. As very few studies have been conducted on the genetic diversity and population connectivity of regional shark populations, this study/chapter aimed at developing novel molecular markers which could be implemented in diversity and population studies on commercially important shark species in South Africa. As the development of molecular markers can be a time consuming and laborious endeavor, Next-Generation Sequencing (NGS) technologies were chosen for the expeditious identification of these markers.

One *Mustelus mustelus* and two *Carcharhinus obscurus* samples were sent for reduced genome sequencing on a HiSeq Illumina and Ion Proton platform respectively. For *M. mustelus*, approximately 35 GB of data was received containing 51,6 million reads with an average read length of 250 bp whereas *C. obscurus* yielded 5, 2 GB of data containing 27,6 million reads with an average read length of 213 bp. Comparing the different available NGS platforms or sequencing approaches, the 454 pyro-sequencing by Roche delivers the longest

read lengths. These longer reads are for example useful for finding adequate flanking regions during primer design. Longer reads also allow for better detection of unfavorable low-complexity regions which can be filtered out in order to identify regions of interest more efficiently within the reads (Wei *et al*. 2014). In terms of cost however, the 454 Roche platforms are expensive as it makes use of emulsion PCR which requires laborious titration steps in order to link DNA templates to the beads. The Illumina platforms have seen a great reduction in sequencing cost and delivers high sequence throughput. Although these platforms have a greater sequencing capacity in comparison to other platforms, very short reads (300bp for paired-end and 150bp for single-end reads) are produced. It is only when using the Illumina HiSeq and MiSeq platform that longer reads can be produced (up to 300bp for paired-end reads on HiSeq and 600bp for paired-end reads on MiSeq) (Illumina Incorporation 2013). The Ion Torrent can be seen as the middle ground with respects to read quality and length as well as the cost of sequencing for microsatellite development (Wei *et al*. 2014). In order to cost-effectively develop molecular markers whilst still maintaining good read quality, it was decided to pursue both the Illumina HiSeq and Ion Proton platforms for sequencing.

Although both platforms follow a sequence-by-synthesis approach, Illumina HiSeq and Ion Proton differ in the use of their detection devices and chemistry (Mascher *et al*. 2013). For both platforms, restriction enzymes are used to create a reduced genome which can be sequenced. DNA-barcoded adapters are ligated onto restriction enzyme fragmented segments in order to parallel sequence individuals in a single run (Mascher *et al*. 2013). Both platforms have a very different approach with regards to how nucleotide orders are determined within the sequences. The Illumina sequencer has millions of lanes on a flow cell made of glass. Within each lane thousands of oligos are attached to the surface of the flow cell to which adapters on the fragments hybridize. Bridge amplification commences with the building of a complimentary strand onto the bridged fragment, forming a double stranded bridge. Sequencing commences with the wash of fluorescently labeled nucleotides over the flow cell. These nucleotides compete for incorporation into the growing complimentary strand. Once a nucleotide is incorporated into the complimentary strand; it releases a fluorescent signal, which is detected by a light sensitive camera. Hundreds of these fragments are sequenced in parallel with the same reads being grouped into clusters and sequenced simultaneously (Glenn 2011; Fu *et al*. 2013). To ensure that a single nucleotide is incorporated during every cycle, the labels on the nucleotides serve as polymerization terminators. The nucleotide

incorporated into the complementary strand is determined by imaging and the label cleaved off for the following cycle (Mascher *et al*. 2013). With the MiSeq/HiSeq Illumina platform, sequencing can take as little as eight hours and can yield reads lengths from 36bp up to 2 x 150 pair-ended reads for HiSeq and 2 x 250 pair-ended reads for MiSeq which will result in easier contig assembly in comparison to Illumina's previous platforms (Glenn 2011; Fu *et al*. 2013).

The Ion Proton platform is the first commercially available platform that does not require fluorescently labeled nucleotides or light sensitive camera technology in order to sequence. It is cost and time-efficient as sequencing can be accomplished in as little as two hours with library preparation time being less than six hours for eight samples (Glenn 2011; Fu *et al*. 2013). DNA is prepared by shearing the DNA into millions of fragments and hybridizing each fragment onto a bead followed by a PCR step to cover the beads with the same fragment. Sequencing starts with the chip being flooded with one of the four nucleotides. When the nucleotide is incorporated into the complimentary strand, it releases a hydrogen ion which causes a change in the pH of the solution in well. This change in pH is recorded and the nucleotide is identified as the next base within the sequence. Every fifteen seconds a new nucleotide is washed across the chip (Glenn 2011; Fu *et al*. 2013). To determine how many of the same nucleotide was incorporated sequentially, the sensor must detect the degree of the pH change. This can introduce errors into the sequence as the sensor can experience difficulties in assessing the degree of the change in pH when several deoxynucleotide triphosphates (dNTPs) are incorporated in the same cycle. With regards to this the Illumina platform might be less prone to these types of errors as only a single nucleotide is incorporated with each cycle, making it easier to sequence repetitive regions with accuracy (Mascher *et al*. 2013). The Illumina and Ion Proton platforms further differ in their read layout, indexing method as well as sequencing primers. For Illumina platforms, all read lengths are the same and can be sequenced as paired-ended reads whereas the Ion Proton reads differ in length and are only sequenced in single-end reads (Mascher *et al*. 2013). For the Ion Proton, read length is also slightly less than the Illumina platform (up to 300pb for paired-end reads on HiSeq platform) with an average read length of 200 bp (Glenn 2011; Fu *et al*. 2013).

Regardless of the platform used to generate the sequencing data, accurate molecular marker identification relies on both the quality and length of the reads produced. (Wei *et al*. 2014). When comparing the data from the two different platforms the HiSeq platform produced an

average read length of 250bp, whereas the Proton platform yielded average read lengths of 213bp. Both the *M. mustelus* and *C. obscurus* reads were filtered for poor quality reads, leaving only reads of good quality to construct contigs with. For *M. mustelus,* the largest contigs assembled were 1 200bp whereas the largest contigs for *C. obscurus* were only 370bp. The noticeable difference in contig size could be attributed in the difference of average read length as well as the amount of reads from which the contigs could be constructed. When taking into consideration that the HiSeq platform produced 18 GB of usable reads in comparison to the 5,2 GB the Ion Proton yielded as well as the difference in average read length that the two platforms produced, it is not surprising that the contigs constructed were significantly different in size. It also has to be considered that the Illumina run was performed aiming for higher genome coverage (x10 times at least) with the one *M. mustelus* sample in comparison to the two *C. obscurus* samples that were run on the Ion Proton platform using a RAD sequencing approach without any specific coverage in aim. For the Ion Proton runs, it is also interesting to note the considerable difference in sequencing data obtained from different library preparations. The same *C. obscurus* sample produced twice the amount of reads when digested for 90 minutes (18 million reads) in comparison to the longer digest of 180 minutes (8 million reads). It is possible that the restriction enzymes reduced the DNA to such small fragments during the longer digest, reducing the actual number of fragments that could be sequenced.

From the constructed contigs, perfect microsatellite regions were identified within both *M. mustelus* and *C. obscurus*. *Mustelus mustelus* contigs yielded more candidate microsatellite regions (2 700) in comparison to that of *C. obscurus* (1 255) and the amount of usable microsatellite regions (di-,tri- and tetra-nucleotide repeats) were considerably more in *M. mustelus* (1 890) than in *C. obscurus* (170). This could once again be attributed to the variation in contig size that was constructed for both species as well as the initial amount of sequencing data received for *M. mustelus* in comparison to *C. obscurus*. Although it is possible to isolate microsatellite regions from raw reads without constructing contigs, longer contigs provide an obvious improved ability to detect polymorphism within the compiled reads. This in turn reduces the amount of redundant microsatellite regions identified due to lower sequence confidence (Zalapa *et al*. 2012).

For *M. mustelus* and *C. obscurus*, 15 and 14 microsatellite regions were selected for primer design, respectively. Only microsatellite repeat regions located in the middle of the contig and which aligned to previously identified microsatellites in other shark or marine species

were considered for primer design. For *M. mustelus*, all 15 microsatellite primer pairs amplified successfully, whereas only eight out of 14 microsatellite primer pairs successfully amplified for *C. obscurus*. It is possible that the quality of the contigs used to construct primers for each identified microsatellite region played a role in the amplification success of the downstream testing. As *M. mustelus* contigs were larger and constructed from longer reads in comparison to that of the *C. obscurus* reads, it is possible that this affected the quality and specificity of the forward and reverse primers designed for each species. In a very preliminary attempt to test for polymorphisms within the successfully amplified markers, a higher resolution agarose gel was used. Although some differences in fragment sizes can be seen for some of the loci tested, indicating polymorphism, these candidate loci should further be validated using polyacrylamide gel electrophoresis. Fluorescently labelled primers will then be obtained for the markers that show intra-species polymorphism. Furthermore, basic diversity estimates such as observed and expected heterozygosity as well as polymorphic information content will have to be determined for these markers in order to assess their usefulness in population and other related genetic studies.

Since no reference genome is available for *M. mustelus* or *C. obscurus*, the reduced genome of the ghost shark *Callorhinchus milii*, estimated to be approximately 910 Mb long (Venkatesh *et al*. 2007), was downloaded in order to map the reads from both species. The *C. milii* genome was used as a proxy for the expected coverage of the *M. mustelus* genome. Although both species successfully mapped to the *C. milii* genome, only *M. mustelus* yielded possible SNP sites. For *M. mustelus*, 767 SNP sites were identified, of which 256 were heterozygous SNPs and 19 were multi-allelic SNP sites. When looking at the frequency of substitutions, Adenine was mostly substituted by Guanine and Cytosine, whereas Cytosine was mostly substituted by Guanine and Guanine by Cytosine. The largest amount of multi-allelic SNPs (37%) contained Guanine and Cytosine. Several factors could have contributed to the unsuccessful identification of possible SNP sites for *C. obscurus*. The reduced genome obtained from *C. milii* contained only unplaced scaffolds to which *C. obscurus* reads were then mapped. The amount of read data for the *C. obscurus* files contained 5,2GB of data whereas the amount of data contained within the *C. milii* scaffolds only amounted to 945 Mb. It is therefore quite possible that there was not a sufficient amount of reads within both species data files to adequately map *C. obscurus* to *C. milii*. In comparison to *C. obscurus* files (5,2 GB), *M. mustelus* files contained three times the amount of data (18 GB). The increase in data contained within the *M. mustelus* files might have made it possible for the *M.*

mustelus reads to be mapped successfully against the *C. milii* scaffolds as both *M. mustelus* and *C. obscurus* species are not as closely related to *C. milii* on an evolutionary scale (see Figure 3.2).

Although *M. mustelus* contigs contained considerably more read data (18 GB) in comparison to the amount of data for *C. obscurus* (15 GB), the amount of SNP sites isolated from mapping the *M. mustelus* to *C. milii* (767) grossly underperforms when compared to the thousands of SNP sites that are usually isolated during mapping. Ideally samples of the same species would be mapped against each other once a preliminary reference genome had been constructed from a sample sequenced at high coverage. In terms of SNP identification, this study was therefore at a disadvantage as contigs had to be mapped to sequencing data from a different shark species with extremely low coverage. This was further aggravated by the smaller contigs that were constructed for *C. obscurus* in comparison to those of *M. mustelus*. Figure 3.2 shows the evolutionary distance between the reference species *C. milii* (subclass Holocephali) and the two mapped species, *M. mustelus* and *C. obscurus* (subclass Elasmobranchii). It is therefore possible that *M. mustelus* and *C. obscurus* mapped poorly to the scaffolds of *C. milii* simply due to the large evolutionary distance between the two subclasses.

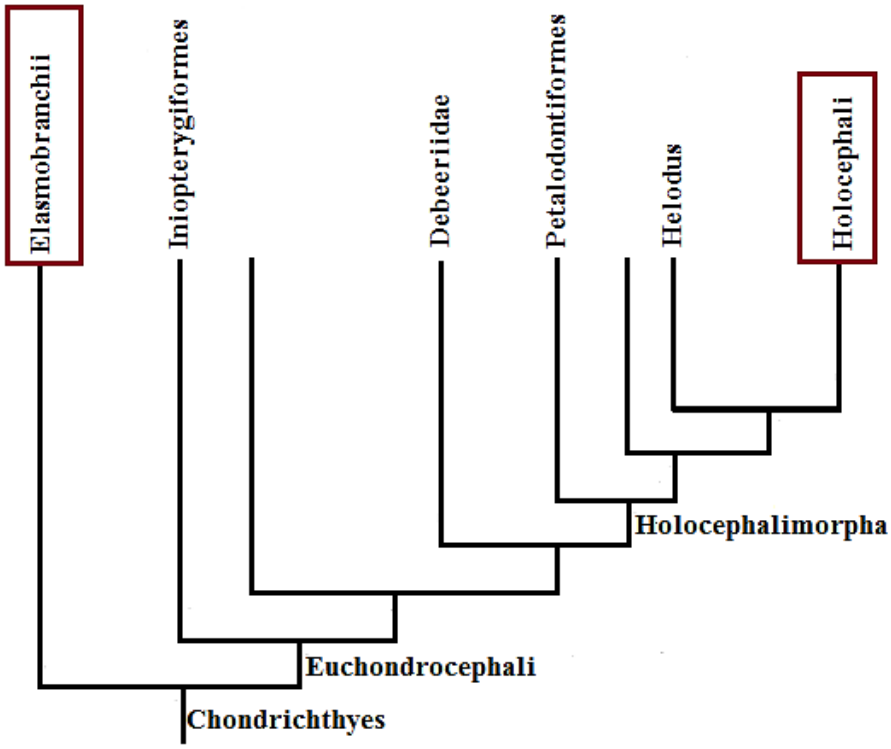

Figure 3.2: Evolutionary distance between the reference species *C. milii* (subclass Holocephali) and the two mapped species, *M. mustelus* and *C. obscurus* (subclass Elasmobranchii) Adapted from Lund & Grogan (1997).

3.5 CONCLUSION

This study provided the first reduced genome sequences for *M. mustelus* and *C. obscurus*. For *M. mustelus*, a total of 51 599 112 reads with an average length of 250bp were obtained using the HiSeq Illumina platform. For *C. obscurus*, a total of 27 685 455 reads with an average length of 213bp were obtained using the Ion Proton platform. For *C. obscurus*, a digestion time of 90 minutes yielded twice the amount of sequencing data in comparison to a digestion time of 180 minutes. For both species, contigs were constructed in order to identify potential microsatellite regions of which 15 regions for *M. mustelus* and 14 regions for *C. obscurus* were selected for primer design. All *M. mustelus* primers and eight *C. obscurus* primers amplified successfully. This could be attributed to the larger contigs that could be constructed for *M. mustelus* in comparison to that of *C. obscurus*. Both species were mapped against the *C. milii* genome in order to identify possible SNP sites. SNP sites could not be successfully identified for *C. obscurus*, whereas *M. mustelus* yielded only 767 SNP sites. This can be attributed to the large genetic distance between the reference species and *M. mustelus* and *C.*

obscurus, the reduced genome that was available for the reference species as well as the quality of the contigs that were mapped to the reference genome. For future studies, the identified microsatellite markers will need to be further validated and characterised by genotyping. For the few candidate SNPs identified, primers will also need to be designed in order to validate SNP regions using genotyping. It would however, be more advantageous to sequence more *M. mustelus* and *C. obscurus* samples in order to map them to the already existing *M. mustelus* and *C. obscurus* sequences. Preliminary sequences could therefore serve as reference sequences which might increase the probability of identifying possible SNP sites for both species. Once validated, the microsatellite and SNPs identified within this study could be used in various downstream applications such as assessing the genetic diversity, stock structure and reproductive strategy (such as polyandry and multiple paternity). This in turn could contribute to an integrated approach to management and conservation of regional *M. mustelus* and *C. obscurus*.

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CHAPTER 4

In conclusion

4.1 INTRODUCTION

Since the 1920s, elasmobranchs have been commercially fished, resulting in a drastic decline in numbers and increasing the risk of extinction for several shark populations. The overall decline in numbers can however be attributed to several factors. Firstly, sharks are being commercially fished in the same way that teleost fishes are fished although they follow a different life history strategy (Musick *et al*. 2000; Stevens *et al*. 2000). In comparison to teleost fishes, sharks reached sexual maturity at a later age, are less fertile, produce less young and have longer gestation periods. This K selected life history strategy makes it impossible for population numbers to be replaced as speedily as in the case of teleost fishes (Simpfendorfer *et al*. 2011; Dulvy *et al*. 2013). Secondly, sharks are generally not fished on a species-specific basis. Most shark species end up as by-catch and are then lumped into large familial groups. This compromises species-specific catch recordings (da Silva *et al*. 2015) and poses a problem for management strategies to be implemented successfully without knowing how many individuals are being taken annually. Lastly, in economic terms, sharks have a lower value than teleost fishes and need to be fished at a larger extent than the teleosts (da Silva *et al*. 2015). Globally, governing bodies have realised the urgency for management steps to be taken to ensure sustainable utilization of shark populations (Stevens *et al*. 2000; Worm *et al*. 2013). This however, proved to be difficult as several species were data deficient and existing studies on for example behaviour and movement did not provide any information on the species ability to respond to environmental changes. Consequently, molecular assessment of shark species increased, with the specific aim of assessing stock structure, genetic diversity and mating behaviour within different shark species (O'Connell & Wright 1997).

As studies on these aspects of sharks progressed, it became evident that shark species also differed significantly in their reproductive strategies (Griffiths *et al*. 2012). Several shark species seemed to engage in polyandrous behaviour which increased the potential for the presence of multiple paternity. Although the direct and indirect benefits of multiple paternity is not yet known, the presence of multiple paternity within a population could possibly affect the genetic diversity of the population and therefore needs be taken into account for management of specific populations (Daly-Engel *et al*. 2007). For most genetic studies, it is

too costly and time consuming to develop species-specific markers and several studies thus far has opted to cross-amplify already developed molecular markers to closely related species. Fortunately, with the technological advances and cost reduction in molecular marker development, this is gradually changing and it is becoming more feasible to develop species-specific markers.

This study aimed to increase the growing amount of molecular genetic data on three commercially important shark species *Mustelus mustelus*, *Carcharhinus obscurus* and *Sphyrna lewini* which could be integrated into novel or existing management strategies. In particular, the study aimed to investigate molecular aspects of the mating strategies of these sharks as well as develop species-specific markers for the species for which currently no molecular markers exists. These markers, if proven to be polymorphic, could be applied in several other population genetic studies of these and other closely-related sharks targeted by regional fisheries.

4.2 ASSESSMENT OF MULTIPLE PATERNITY

The first aim of the study was achieved through the cross-amplification of already developed molecular markers to the three study species. Marker panels of 22 microsatellite markers were initially tested on four litters of each species and then reduced to five or six markers within a panel for each species respectively. Litters and their mothers were genotyped and analysed for the presence of multiple paternity within the species. Multiple paternity was established within all three species with *M. mustelus* having the highest frequency of MP (67%) followed by *S. lewini* (46%) and *C. obscurus* (35%). The frequency of MP was similar to the MP frequency previously reported for closely related species. What is however interesting is that the South African *M. mustelus* had a considerable higher frequency of MP (67%), in comparison to *M. mustelus* of the Adriatic Sea (47%) (Marino *et al*. 2015). This once again highlights the many different factors that can contribute to the variation in MP rate observed between and within species.

Although it is thought that MP influences the genetic diversity within a population, there is still uncertainty regarding the reason behind polyandrous mating (Daly-Engel *et al*. 2006, 2010; Boomer *et al*. 2013). It is theorised that polyandrous mating is rather a consequence of mate encounter rate as well as sexual conflict avoidance (Daly-Engel *et al*. 2006; 2010). The more sharks that frequent a specific area, the more likely males and females are to encounter one another and mate. Although female sharks are larger than males in some species, it

becomes increasingly difficult for the female to ward off possible suitors when male sharks aggregate to mate. Attempting to fight off aggregating males can lead to serious physical injury to the female and in these cases it is in the female's best interest to copulate and avoid unnecessary physical injury (Neff & Pitcher 2002; Daly-Engel *et al*. 2006, 2010). Another phenomenon which can lead to multiple paternity is the ability of some shark species to store viable sperm within their shell glands (Saville *et al*. 2002; Chapman *et al*. 2004; Daly-Engel *et al*. 2006; Byrne & Avise 2012). Both *C. obscurus* and *S. lewini* have been found to store viable sperm (Pratt 1993) and it is quite possible that *M. mustelus* is capable of storing sperm (Hamlett *et al*. 2002; Storrie *et al*. 2008; Farrell *et al*. 2010). The frequency of MP can therefore be influenced not only by mate encounter rate but by the occurrence of polyandrous behaviour, ability to store sperm as well as reproductive mode differences between shark species. Nonetheless, it is important to include this information on mating behaviour into regional management strategies. Most importantly, the variation found between species and even between populations of the same species should be considered for a more species-specific approach to fisheries management (Bester-van der Merwe & Gledhill 2015).

4.3 IDENTIFICATION OF SPECIES-SPECIFIC MOLECULAR MARKERS

The second aim of the study entailed the identification of novel molecular markers for *M. mustelus* and *C. obscurus*. Species were sent for reduced genome sequencing on Hiseq Illumina and Ion Proton platforms. For *M. mustelus*, 18 GB of data was obtained from the HiSeq Illumina platform, whereas 5,2 GB of *C. obscurus* data was obtained from the Ion Proton platform. The difference in the amount of data obtained could mostly be attributed to the difference in library preparation and consequently the variance in fragment size produced by the two platforms. For the HiSeq platform, fragment shearing was performed using a single restriction enzyme and paired-end sequencing approach whereas for the Ion Proton library, fragment shearing was conducted using two restriction enzymes (*EcoR1* and *Mse1*) followed by size selection of fragments between 200-300 bp. It is possible that a majority of the Ion Proton reads were smaller than 150bp with a high percentage deemed too short for further analysis.

From the data, contigs were constructed from which perfect repeat microsatellite regions were identified for both species. Fifteen microsatellite regions were selected from the *M. mustelus* data set and 14 microsatellite regions from the *C. obscurus* data set and primers were designed for both species. For *M. mustelus*, preliminary testing showed successful

amplification for all 15 primer pairs. For *C. obscurus*, only eight primer pairs amplified successfully during preliminary amplification. As no reference genome exists for either of the study species, scaffolds from the Australian ghost shark *Callorhinchus milii*, was used for mapping of both sequencing data sets. Both species mapped successfully to *C. milli* but possible single nucleotide polymorphisms could only be identified for *M. mustelus* (767 SNP regions). Both the study species fall under the subclass of Elasmobranchii whereas *C. milii* is classified as Holocephali. It is possible that the mapping of sequencing reads to the *C. milli* scaffolds was not optimal given the large evolutionary distance between the two classes of elasmobranchs. Another factor that certainly played a role was the restricted amount of *C. milii* data that was available to map the current data sets against. For *M. mustelus*, 2 700 microsatellite regions and 767 SNP regions were identified whereas for *C. obscurus*, 1 255 microsatellite regions were identified. Microsatellite regions obtained for both *M. mustelus* and *C. obscurus* are comparable to other that of other microsatellite identification studies using NGS platforms. For example, 1 344 microsatellite regions were isolated for the tope shark *Galeorhinus galeus* (Chabot & Nigenda 2011), 3 905 microsatellite regions for Scaleshell *Leptodea leptodon* (O'Bryhim *et al*. 2012), 1 590 microsatellite regions for Julimes pupfish *Cyprinodon julimes* (Carson *et al*. 2013) and 749 microsatellite regions were isolated for the ringed salamander *Ambystoma annulatum* (Peterman *et al*. 2013).

4.4 FUTURE WORK AND STUDY LIMITATIONS

For future work, it would be beneficial to collect more litter samples from all three study species from different locations along the coast of South Africa in order to compare whether there is any significant difference in the frequency of MP found amongst different populations of the same species. If populations differ in MP frequency, it will need to be assessed whether fisheries have had any effect on the mating behaviour of these populations in both non-fished as well as heavily fished areas (Bester-van der Merwe & Gledhill 2015). Although preliminary testing has shown microsatellite primer amplification for both species-specific developed microsatellite primers, validation for these markers still needs to be performed. All 29 markers will need to be tested for polymorphism and characterised within a subset of individuals through genotyping. If any markers do show a high polymorphism, a further study could entail applying these markers in genetic diversity and population genetic studies. A recent study by Maduna *et al*. (in review) used cross-amplified microsatellite markers from closely related species in order to assess genetic diversity and population connectivity within *M. mustelus* along the South African coasline. It would be beneficial to

genotype the same populations with the species-specific microsatellite markers in order to elucidate whether there is a finer level of structure visible within the populations and whether these species-specific markers influence the outcome of genetic diversity assessment. For the SNP regions isolated, not having a reference genome of a closely related species was a major limitation to the study. For future work and SNP identification in particular, it would be beneficial to sequence more individuals for both study species in order to build preliminary reference scaffolds to which other samples can be mapped. This will allow for much better coverage when mapping multiple samples within species and could yield a considerable number of possible SNP regions. With regards to the current data sets, immediate future work could entail gene annotation for preliminary genome characterization and comparative mapping of the two species.

4.5 CONCLUSIONS

Ever since sharks made their appearance 400 million years ago, they have played an important role in the top-down control of oceanic as well as coastal ecosystems (Carrier *et al*. 2010; Biery & Pauly 2012; Dulvy *et al*. 2013). These shark species help to structure marine communities by influencing the behaviour and mortality rates of the meso-consumers within the ecosystem (Molina & Cooke 2012). Removing these larger apex predators could lead to a disruption of both ecosystems and linefish fisheries as the knock-on effect could result in a change of the prey community composition (Stevens *et al*. 2000; Carrier *et al*. 2010; Worm *et al*. 2013). By removing apex predators from the ecosystem, prey communities could flourish and exhaust their food resources, disrupting the balance of the food web. Even the discard of remains after shark finning has an effect on the amount of available food for scavenging species (Stevens *et al*. 2000). Apart from affecting marine ecosystems, the removal or loss of shark populations can have a socio-economical effect as many communities from developing and developed countries are dependent on shark fisheries as a source of income. A decline in these resources would lead to even more fishing pressure being placed on the already limited fish resources (Simpfendorfer *et al*. 2011).

It is therefore crucial to evaluate each and every shark population affected by fishing pressure to assure that these populations remain sustainable for fisheries. In order to best manage shark populations, data on genetic diversity, stock structure and mating strategy needs to be collected and evaluated. This will ensure that already genetically inbred or weak populations (which would not be able to rebound as quickly from extensive removal of individuals) are

protected rather than further exploited. Data on the genetic diversity of a population is also crucial in order to determine how many individuals of a population can be fished before population decline sets in. By knowing how well a population can adapt to the fluctuating environment, fishing quotas on populations can be implemented to ensure sustainable fisheries of these populations. This study therefore aimed to increase data on the mating strategies of three commercially important shark species in South Africa (as MP can have an effect on genetic diversity of a population) as well as to identify possible novel molecular markers with which population structure and genetic diversity of our local populations can be evaluated in future.

In summary, this study provided the first evidence for the presence of MP in the three commercially important shark species within South Africa and provided the first possible novel molecular markers for both the *M. mustelus* and *C. obscurus* species. The study has added valuable data on the mating strategies of South African *M. mustelus*, *C. obscurus* and *S. lewini* shark populations. In addition, the potential molecular markers identified here could be applied in various down-stream assessments of regional populations on a molecular level. This could assist in the establishment and even implementation of more effective management programs for these and other closely-related shark species affected by regional fisheries. Both the South African Draft Biodiversity Management Plan (RSA 2014) and the South African National Plan of Action (NPOA) for Sharks (DAFF 2014) have stressed the need to employ genetics in assisting with molecular species identification and stock structure analysis.

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APPENDIX A

Table 3.3: Primer sequences for 15 *M. mustelus* and 14 *C. obscurus* microsatellite regions including contig ID, product size, melting temperature (Tm), percentage of GC content, maximum intra-pair difference (anv) as well as maximum self-complementarity score (3').

	ID	Primer	Start in contig	Length (bp)	PCR Product size (bp)	Tm	GC%	anv	3'	Primer sequence (5'-3')
M. mustelus	603691_889_5405 (MM0001)	F:	507	20	210	60	45	8	3	CCCCATTTGCAAACAGAGTT
		R:	716	20		60	45	5	2	ATTTCCCGCTGTTACATTGC
	17731961_828_2935 (MM0002)	F:	661	20	163	60	50	6	2	TTGTCTGCAGGAAACACAGC
		R:	823	20		60	45	3	2	GCATCGTGTGAAATGGGAAT
	19222092_770_1754 (MM0003)	F:	157	20	240	60	55	5	3	ATACACGGACCGACTCGAAC
		R:	396	20		60	50	4	2	TAATGCCGAGATCAGGAACC
	26403340_740_2107 (MM0004)	F:	358	20	173	60	50	4	1	TCCATCCAGCGTTAAAGGAC
		R:	530	20		60	50	4	2	GCACCAGAGCTTCCCATTTA
	12961358707 1755 (MM0005)	F:	262	20	282	60	60	6	2	ACCACTCCCTGCAGCACTAC
		R:	543	20		60	50	3	2	AGGAGATGCTTTGGCACTTG
	14682365_1857_7998 (MM0006)	F:	1461	20	212	60	60	4	1	CACCGGAGACCTCTAACTGG
		R:	1672	20		60	50	4	2	CGATGATGATGAAGGACGTG
	18961995_1089_3100 (MM0007)	F:	809	20	219	60	50	3	3	TCCCTCATTTGCTTCAGGAG
		R:	1027	20		60	45	4	0	CGACATGAAACGCAGAAAGA
	26836431_1069_3092 (MM0008)	F:	281	20	431	60	50	6	3	AGTAAGGCGCGCTATGATTG
		R:	711	20		60	55	3	0	TAGAAGTCATCGCCCTCCAC

	7951092 (MM009)	F:	230	20	172	60	50	7	2	ACGGTTCTGAGCAATCGTCT
		R:	401	20		60	45	4	3	TGCGATATTCGTCAGGTGAA
	11748443 (MM010)	F:	178	20	299	60	55	6	0	AATCCTGAGCACCAGGACAC
		R:	476	20		60	45	6	1	TGTGTGAATTCCCCAGATGA
	61216 (MM011)	F:	301	20	211	60	50	6	2	ATCTTGTTAACCGCCGACAG
		R:	511	20		60	45	4	1	CGCCATGTTGATCGAAGTAA
	1178354 (MM012)	F:	82	20	208	60	55	3	2	GAGCAGCCAAGCATTAGTCC
		R:	289	20		60	45	5	3	CGGCTTCAGAAATTGGAATC
	14447036 (MM013)	F:	33	20	112	60	50	2	1	TCATTCCTCACACCCACTCA
		R:	144	20		60	50	5	0	AGATCCAGGAGCGAAGAACA
	12929751 (MM014)	F:	181	20	186	60	50	2	1	ACCGCTTGCTTCTGTTGAGT
		R:	366	20		60	50	5	0	TCGCACAGACTGATTGAAGG
	14824632 MM015	F:	283	20	173	60	55	6	2	CACCTGATTGAGCAGGAGGT
		R:	455	20		60	50	4	2	TATGGAGGTTGGGATTGCAG
C. obscurus	45LXA:10554:09194 CO001	F:	106	20	167	60	55	4	1	GGGCCTGTTTGTGCTGTAGT
		R:	272	19		60	57	4	2	GTTCCTTCCAAGCCG

									TCTC
45LXA:09263:12443CO002	F:	19	19	219	60	52	3	0	ATTCGGACTGTGGGAGGAA
	R:	237	20		60	55	4	2	GGGATCGTTCTGGGTGAGTA
45LXA:03070:04813CO003	F:	18	21	177	60	47	3	3	TTCCGTATCCATCCTGTCAAG
	R:	194	21		60	42	5	1	GCATTTAGAAGCAATGCAGGA
45LXA:04796:12627CO004	F:	38	20	165	60	55	3	0	GGTGTTGAGAGGCTGTTTCC
	R:	202	20		60	50	6	0	AAGGCCTGTTTCTGTGCTGT
45LXA:07096:09089CO005	F:	31	20	160	60	50	5	0	CTGTAACACCGGGGAAAAGA
	R:	190	20		60	55	3	0	ACGGATGTGGTCCTGCTTAC
45LXA:05232:05820CO006	F:	33	21	172	60	52	8	2	GATGGAGGTTCTCCTCCAAAG
	R:	204	20		60	55	2	0	CAGGTGAGGGGAAACAAGAG
45LXA:09892:10714CO007	F:	74	20	151	60	55	4	0	TGAGGCCACTTGCTCCTATC
	R:	224	20		60	55	5	2	GATGAGGCATCCTGCTAAGG
45LXA:06987:06961CO008	F:	20	20	155	60	45	6	0	TAGGCTGGAAATCTCCCAAA
	R:	174	20		60	55	4	2	GCCCTGCTCACAGCTTTATC
45LXA:06809:09492CO009	F:	57	20	169	60	60	7	1	GCTCCTAATAGGGCCCAGAC
	R:	225	20		60	40	3	2	AAAAGGAAGGGCAAATCCAT
45LXA:07901:08045CO010	F:	5	22	166	60	36	4	0	CATGGAAAACAAAAACGACAGA
	R:	170	20		60	55	5	1	GGGGCTTGTCAGCCTTTAGT
45LXA:04	F:	42	20	213	60	50	3	0	TGCTGAGGGTAATG

481:12308									CTGTTG
CO011	R:	254	20		60	55	4	1	GACCCCTTGGGAACT CAACT
45LXA:04	F:	87	20		60	50	3	0	CTTTTCCAACTCGGC
849:07121				171					TCTTG
CO012	R:	257	20		60	50	4	1	ATGCTCATTCCTCCA ACCAG
45LXA:02	F:	84	20		60	50	3	3	GAATGTCCAAACTG
939:05771				150					GCACCT
CO013	R:	233	20		60	50	6	0	GGCAAAGGCCTGTTT CTATG
45LXA:09	F:	25	20		60	45	2	0	CTCCTTCCCAAAATC
925:04970				233					CACAA
CO014	R:	257	20		60	60	7	3	CAGAAGGAGAGCCT CTGGTG